UNITED NATIONS

SECRETARIAT

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ST/SOA/SER.J/11
31 July 1974

Original: ENGLISH

SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM 1/

No. 11

DETERMINATION OF THEBAINE IN PAPAVER BRACTEATUM 2/

by: R. Salkin 3/

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Research Division,
Orange, N.J. 07050.
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1/ General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

2/ In the report of the Second Working Group on Papaver bracteatum (document ST/SOA/SER.J/2), it was recommended that methods for the determination of thebaine in Papaver bracteatum should be compared and evaluated; to achieve this, it was decided that participants in the Working Group would forward their methods for publication in this series so that these could be tried out in the various laboratories.

3/ This paper was submitted by Dr. Bernard B. Brown, Director of Research, S.B. Penick and Company.

GE. 74-8087

DETERMINATION OF THEBAINE IN PAPAVER BRACTEATUM UNB-17 4/

The milled crude material is extracted at 40° C with a 50/50 water-methanol mixture adjusted to 0.5 N with acetic acid. The methanol is removed in vacuo and the aqueous residue back washed with ether. The acid water is alkalinized to pH 9.5 in the presence of chloroform and the chloroform extract evaporated to dryness in vacuo. The bases so obtained are assayed by G.L.C. as described in J. Chromatography 89, 76-9 (1974).

Sample UNB-17 yielded 3.7% total bases with a G.L.C. purity of 90%, equivalent to 3.3% anhydrous thebaine alkaloid on the sample as received.

These bases afforded a 2.8% primary yield of pure thebaine, MP 194-196° C, whose infrared spectrum (Nujol mull) matched an authentic sample.

4/ The method is applicable to all plant parts either wet or dry, and with minor modifications, can be used for the recovery of micro quantitites of alkaloids.

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20 August 1974

ENGLISH
Original: FRENCH

SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM[1/]

No. 12

DETERMINATION OF THE THEBAINE CONTENT OF PAPAVER BRACTEATUM[2/]

by:[3/] J.-M. Pelt and M. Lorrain,

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1/ General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

2/ In the report of the Second Working Group on Papaver bracteatum (ST/SOA/SER.J/2), it was recommended that methods for the determination of thebaine in Papaver bracteatum should be compared and evaluated; to achieve this, it was decided that participants in the Working Group would forward their methods for publication in this series so that these could be tried out in the various laboratories.

3/ This paper was submitted by Mr. C. Candillon, Director of the Agricultural Department, Francopavot.

GE.74-8743

The method used for separating thebaine from the various poppy alkaloids was based on one recommended by Pfeifer.[4/] This process enables thebaine to be isolated in the form of the tartrate.

I. Separation of the principal poppy alkaloids

The dry sample (roots, leaves or flowering tops) is reduced to a powder, which is ground in a mortar with a 5 per cent sodium carbonate solution. Five ml of alkaline solution are used for 10 g of dry powder.

After saturation of the powder, complete extraction of the alkaloids is effected by mixing with: methylene chloride:ether:methanol: 60:30:10 (V/V). Extraction continues for one hour. After filtration, the residue is extracted a second time. The extracts are combined and evaporated under reduced pressure. The dry residue is taken up with ether. This operation separates the ether-soluble pigments from the alkaloids, which are converted into salts by a solution of N/2 sulphuric acid. Separation is continued by the addition of aqueous ammonia (pH 7 to 8) to the aqueous acid phase. The alkaloid bases are then extracted with chloroform; 0.1 N sodium hydroxide is added to this organic phase, thus eliminating a few more pigments.

The alkaline phases have extracted morphine, narceine and narcotoline. These alkaloids were not detected in the various poppies studied: Papaver bracteatum and P. orientale. However, these basic solutions extract many pigments, thus justifying this procedure.

The thebaine base in chloroform solution is extracted by an 0.2 per cent tartaric acid solution. Under these conditions, codeine is also extracted in the form of tartrate, but the poppies studied only contain traces of codeine.

After collecting the aqueous acid phase, the thebaine base is extracted in chloroform in an ammoniacal medium. After separation and filtration through Whatman paper, the organic phase is dried under reduced pressure. The residue is subjected to chromatographic analysis and quantitative determination.

4/ S. Pfeifer, Bull. Narcot., 1958, X, No. 3, p.18.

II. Chromatographic analysis of the extract

The method of ascending chromatography on a thin layer of silica gel H is used. The solvent consists of the following mixture: toluene, acetone, methanol, 25 per cent ammonia: 45:45:7:3 (V/V). Migration takes place in 30 minutes. The solvent front is 10 cm.[5/]

The detecting agents are Dragendorff's reagent and concentrated sulphuric acid. When the latter is sprayed on the chromatogram thebaine appears as yellow-brown, while the other alkaloids remain invisible when cold, the colour being developed only after the plate has been placed in an oven at 100°C for 20 minutes.

Under these conditions, the thebaine has an Rf value of 0.45 to 0.50. Its amount as a proportion of the total alkaloid content is determined after spraying the chromatogram with Dragendorff's reagent and visual comparison of the thebaine spot with reference solutions for thebaine, developed under the same conditions.

Thebaine was found to be the main alkaloid in several samples analysed; on the other hand, a mixture of three or four alkaloids is very often found, in which thebaine represents only 30 to 50 per cent of the total alkaloid content.

III. Quantitative determination

The total quantity of alkaloids is determined by acidimetry.[6/] The results are expressed as the weight of thebaine per 100 g of dry plant.

Determination is carried out on the dry residue of the extract. The alkaloids are dissolved in a known excess of 0.02 N, say N ml hydrochloric acid, in the presence of methyl red. A solution of 0.02 N sodium hydroxide is added until the indicator turns yellow, say n ml.

Calculation of the results:

1 ml of 0.02 N sodium hydroxide corresponds to $\frac{311.3}{50}$ mg of thebaine

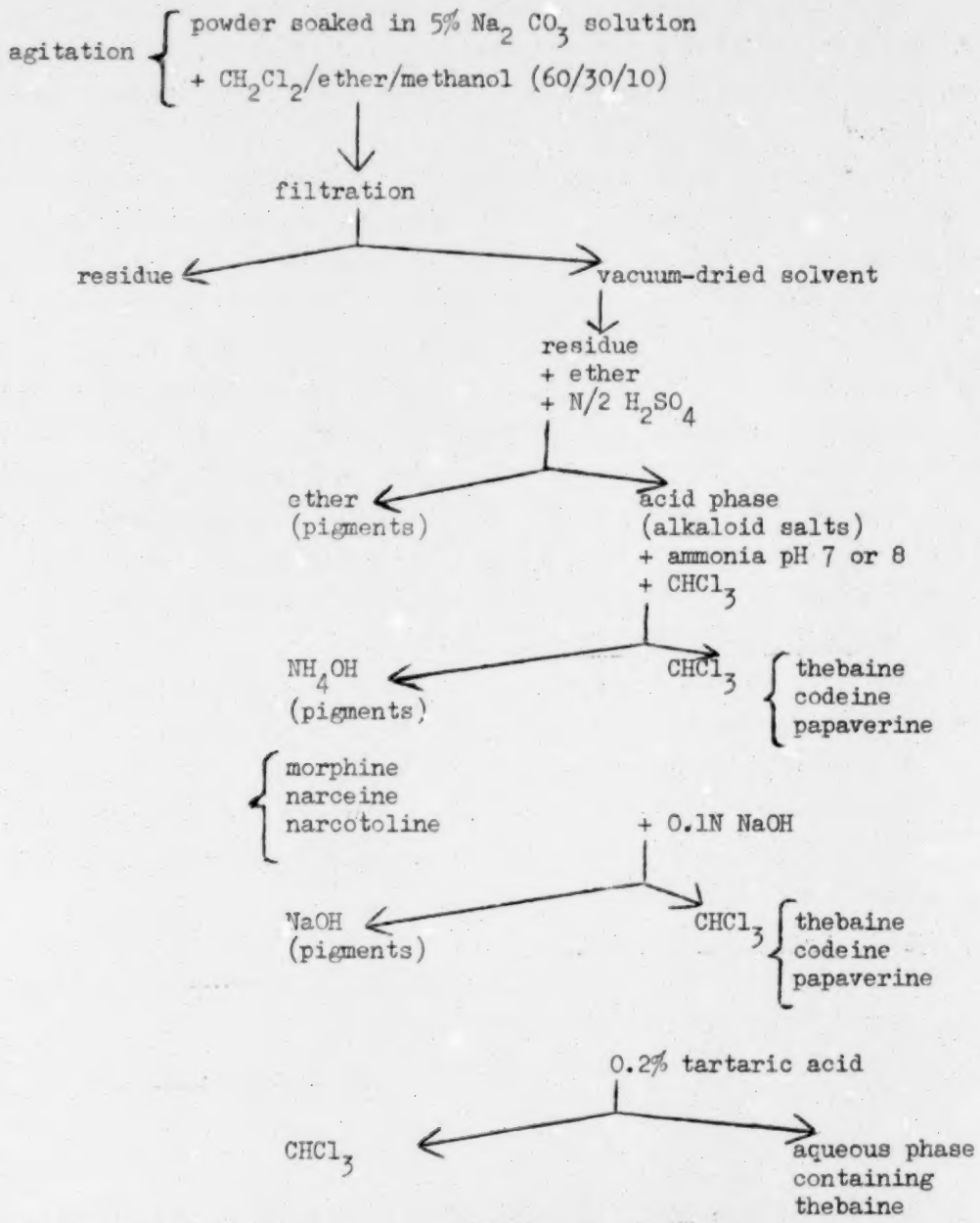
The test sample consists of 10 g of dry plant

Percentage of alkaloids (as thebaine): $\frac{(N-n) \times 311.3 \times 100}{10 \times 50 \times 1000}$

5/ Egon Stahl, Chromatographische und mikroskopische Analyse von Drogen.

6/ Pharmacopée française, VIIIe édition, p.167.

METHOD OF SEPARATING THE PRINCIPAL POPPY ALKALOIDS



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28 August 1974
Original: ENGLISH

SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM 1/

No. 13

ISOTOPE DILUTION ASSAY OF THEBAINE IN PAPAVER BRACTEATUM 2/

by: K.C. Reid, 3/

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1/ General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

2/ In the report of the Second Working Group on Papaver bracteatum (document ST/SOA/SER.J/2), it was recommended that methods for the determination of thebaine in Papaver bracteatum should be compared and evaluated; to achieve this, it was decided that participants in the Working Group would forward their methods for publication in this series so that these could be tried out in the various laboratories.

3/ This paper was submitted by Dr. E.L. Grew, Research and Development, Macfarlan Smith Ltd.

ISOTOPE DILUTION ASSAY OF THEBAINE IN P. BRACTEATUM

28.8680 g of the sample of P. bracteatum (UNB-17, as received, finely ground), 0.5357 g of C^{14}-N-methyl labelled radioactive thebaine alkaloid[4/], 150 ml of acetone, 5 g of calcium hydroxide and 15 ml of water were vigorously stirred together for 3 hours at room temperature in order to obtain uniform mixing of the active and inactive forms of the alkaloid present. The mixture was then filtered under vacuum and the filter cake washed with 100 ml of acetone. The combined filtrate and washings were evaporated to dryness in a rotary vacuum evaporator at a water-bath temperature of 40°C and the residue dissolved in chloroform (ca 100 ml). The chloroform solution was filtered to remove insoluble matter and then evaporated to ca 30 ml under reduced pressure (rotary vacuum evaporator). The resulting concentrate was stirred vigorously with 5 ml of 50% w/v tartaric acid solution for 30 minutes and the thebaine acid tartrate which crystallised out filtered off and washed several times with small portions of fresh chloroform. The crystals were dried, suspended in 10 ml of water, the mixture stirred and dilute ammonium hydroxide solution added dropwise to dissolve the solid and give a final pH of 5.0 to 6.0. One drop of a 10% aqueous solution of disodium ethylene diamine tetra-acetic acid was added followed by 0.2 g of powdered charcoal and the mixture stirred for 5 minutes and then filtered. The clear solution obtained was treated by dropwise addition of an excess of dilute ammonium hydroxide (final pH 9.0 - 9.5) with vigorous stirring in order to precipitate thebaine alkaloid. The latter was filtered off, washed well with water, and dried at 50°C. The material obtained had a melting-point of 192° - 193°C and TLC showed only a single spot with the same Rf value as thebaine, indicating that the sample was fairly pure. It can be further purified if required (and providing sufficient material is available) by recrystallisation from alcohol. The activity of the thebaine isolated was determined by planchet counting of the solid (infinitely thick layer technique) using Panax Geiger counting equipment, but liquid scintillation counting can be employed if preferred. The percentage thebaine in the original sample of P. bracteatum was calculated in the normal way (see below) and found to be 3.2%.

$$\% \text{ thebaine} = 100 \frac{W_L}{W} \left(\frac{C_L}{C_S} - 1 \right)$$

where W is the weight of the sample of P. bracteatum used

W_L is the weight of labelled thebaine used

C_L is the corrected count rate of the labelled thebaine

C_S is the corrected count rate of the sample of thebaine isolated.

4/ The labelled thebaine used had a specific activity of approximately 1.5 uC per gramme and was synthesised in the laboratories of Macfarlan Smith Ltd.

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ST/SOA/SER.J/14
2 September 1974

Original: ENGLISH

SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM[1]

No. 14

DETERMINATION OF THE THEBAINE-CONTENT OF LEAVES OF PHYTOTRON-GROWN[2] PAPAVER BRACTEATUM[3] PLANTS

by: F.J.E.M. Küppers and C.A. Salemink,
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State University,
Utrecht, The Netherlands.

H. Greppin,
Laboratory of Plant Physiology,
University of Geneva,
Geneva, Switzerland.

L. Wuarin and G. Meylan,
Laboratory of Agricultural and Horticultural Techniques,
Geneva, Switzerland.

1/ General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

2/ The cultivation of the P. bracteatum plants was carried out in the Laboratory of Plant Physiology, University of Geneva, under the supervision of Professor H. Greppin, in order to establish optimum conditions for in-door growing of P. bracteatum. For comparative purposes, out-door cultures were carried out under the supervision of Mr. G. Meylan and Mr. L. Wuarin.

3/ Origin of the seeds: P. bracteatum Arya I, Professor I. Lalezari, Department of Organic Chemistry, Faculty of Pharmacy, University of Teheran, Iran.

GE.74-9301

Since the publication of our report[4/] on the analysis of Papaver alkaloids and stability of thebaine, several attempts have been made to improve the extraction procedures and the GLC analysis of thebaine. Because of the instability of thebaine in acidic solutions,[4/] we considered it necessary to use basic solvent mixtures in order to avoid decomposition during extraction. An additional advantage of basic solvent mixtures, which extract the alkaloids in the form of tertiary amines, is the possibility of direct injection and analysis in the gas chromatograph.

In general, it is found that the chemical composition of leaves, roots and capsules is different and consequently the extraction procedures for the individual materials should also differ for optimal thebaine and/or total alkaloid extraction. For example, in the case of the leaves, a pre-purification step should be carried out in order to remove from the extract the chlorophylls and fats, which contaminate the injection ports of the GC system. Because of the lack of radioactive labelled thebaine - which would permit the measurement of the loss of thebaine during the pre-purification procedure - the crude chlorophyll-containing extract is presently still injected into the gas chromatograph as such and therefore the injection port has to be cleaned regularly. It is our present experience that particularly the 80-100 mesh size of the stationary phase support, chromosorb G coated with 3 per cent OV-17, provides excellent separation with complete absence of peak-tailing in routine analyses of thebaine-containing extracts. Using the above stationary phase, a low pre-pressure of 1.8 atm and a relatively low column temperature of 240°C, thebaine shows a retention time of about 15 minutes.

Comparison of the standard extraction procedure for leaves with an exhaustive Soxhlet-extraction

The following procedure was used for the routine extraction of air-dried leaf material of phytotron-grown P. bracteatum plants:

1. The material is ground in a mortar to pass through a 20 mesh-sieve.
2. 500 mg of the resulting powder is extracted for 16 hours at 25°C with 25 ml methanol.
3. The methanolic extract is decanted and evaporated to dryness using a rotatory evaporator.
4. The residue is extracted for 30 minutes at 50°C with 10 ml 2 per cent NH_4OH (spec. grav. 0.900) in methanol.
5. The extracts from 3 and 4 are combined, adjusted to a proper volume and analyzed by GLC.

A number of samples were divided in two equal parts. One part was extracted using the procedure described above and the other part was exhaustively extracted in a

---

4/ F.J.E.M. Küppers, R.J.J.Ch. Lousberg, C.A.L. Bercht and C.A. Salemink United Nations document ST/SOA/SER.J/8.

Soxhlet extraction apparatus (extraction mixture: 2 per cent NH_4OH in methanol, duration: 24 hours, number of passages of extraction fluid 300). The results are given in Table I.

Table I

sample nr.	standard extraction % thebaine (A)	Soxhlet extraction % thebaine (B)	$\frac{A}{B}$ x 100%
1	0.165	0.175	94
2	0.505	0.540	93
3	0.340	0.360	94
4	0.550	0.585	94
5	0.225	0.240	93
6	0.085	0.090	94
7	0.280	0.295	95
8	0.105	0.110	95

These results indicate that the "routine extraction" is almost as effective as an exhaustive extraction and, because of the much smaller demand in time, energy and instrumental equipment, this procedure was applied to the phytotron-grown series of P. bracteatum plants. The results in the following tables are not corrected for the above mentioned difference of about 6 per cent.

Comparison of thebaine content determined in leaf material dried according to four different methods

Until now, no uniform method of drying fresh P. bracteatum samples has been used. In order to establish the most suitable way of drying, which would keep the decomposition of thebaine minimal, the following experiments were carried out:

Eleven phytotron-grown P. bracteatum samples, each containing at least five different plants, were divided into five equal parts. The four drying procedures applied to these samples were:

1. Freeze-drying / 24 hours
2. Oven-drying at 105° / 1 hour
3. Oven-drying at 60° / 3 hours
4. Air-drying at 25° / 48 hours

The fifth part of each series was placed in methanol without previous drying. After drying, all plant samples were extracted by the same "routine procedure" and analyzed by GLC. The results are given in table II.

Table II

sample	freeze-drying % theb.	oven 105°/1hr % theb.	oven 60°/3hr % theb.	air 5/ 25°/48hr % theb.	fresh in methanol 6/
1	0.28	0.25	0.14	0.05	0.01
2	0.17	0.15	0.10	0.06	"
3	0.40	0.33	0.23	0.13	"
4	0.24	0.21	0.18	0.14	"
5	0.30	0.26	0.22	0.20	"
6	0.56	0.48	0.41	0.31	"
7	0.22	0.13	0.11	0.11	"
8	0.39	0.32	0.30	0.28	"
9	0.55	0.48	0.44	0.37	"
10	0.12	0.10	0.09	0.08	"
11	0.58	0.55	0.52	0.46	"
mean	0.35	0.29	0.25	0.22	-

From the data in this table, it was concluded that freeze-drying is the most suitable method to be applied for quantitative thebaine analyses of leaf material.

Until now, the phytotron-grown P. bracteatum plants were sent from Geneva to Utrecht by plane without previous drying. It took about 48 hours between harvest and arrival at our Institute. Biological processes, which had been able to decrease the thebaine content, had thus already taken place. Rigorous freeze-drying could not further account for lower percentages of thebaine as found in table II.

Conditions for phytotron cultures of P. bracteatum

In the phytotron, the following schedule was applied:

a. variation of photo-period: 24, 20, 16 and 12 hours/day

b. variation of light intensity: 8000, 6000, 4000 and 2000 lux

During the photo-period the temperature was 20°C and the relative humidity 80 per cent. During the dark period, the temperature was also 20°C and the relative humidity 60 per cent. All plants were cultivated on artificial soil (terralit), nutrition 0.5 per cent Lonzin plus 0.5 per cent Wuxal. The results of these series of experiments are shown in Tables III and IV and in figures 1 and 2.

5/ Additional freeze-drying of this air dried material caused a further loss of 10 per cent in weight.

6/ The extraction time (2 weeks) proved to be too long; virtually all the thebaine decomposed.

The results indicate that, under the phytotron conditions applied, the combination of a photo-period of 24 hours and a light intensity of 6000 lux induces a maximum thebaine production in plants of about 160 days old, whereas a photo-period of 20 hours/6000 lux gives a maximum thebaine content in plants 130 days old. Both 16 and 12 hours of photo-period seem less favourable for thebaine production.
Note: The values, obtained with 102 day old plants (Table III) all originate from one series of harvest. This series was analyzed on a OV-1 column under apparently less favourable conditions and may have yielded too low results.

In addition to thebaine, substantial amounts of at least two other products were observed in every sample. In the gas chromatogram, their relative retention times were 0.77 and 1.44. Further structural analyses of these compounds will be the subject of a forthcoming report.

Table III

	age of plant at harvest (days)	% thebaine	
		photo-period 24 hr/day	photo-period 20 hr/day
photo-intensity 2000 lux	111	0.16	0.06
	131	0.30	0.30
	151	0.22	0.10
	182	0.16	0.17
	202	0.14	0.26
	237	0.17	0.32
photo-intensity 4000 lux	111	0.12	0.17
	131	0.34	0.36
	151	0.26	0.10
	182	0.24	0.14
	202	0.31	0.20
	237	0.27	0.35
photo-intensity 6000 lux	111	0.15	0.32
	131	0.50	0.55
	162	0.55	
	169		0.05
	182	0.49	0.15
	202	0.31	0.33
	209	0.29	
	237		0.38
photo-intensity 8000 lux	111	0.25	0.16
	131	0.49	0.40
	162	0.45	0.29
	182	0.23	0.22
	202	0.21	0.27
	237		0.30

Table IV

	age of plant at harvest (days)	% thebaine	
		photo-period 16 hr/day	photo-period 12 hr/day
photo-intensity 4000 lux	145	0.20	0.14
	169	0.23	0.13
	196	0.09	0.21
	216	0.20	0.19
	223	0.21	0.09
photo-intensity 6000 lux	145	0.27	0.16
	169	0.09	0.12
	196	0.18	0.24
	216	0.28	0.20
	223	0.15	
photo-intensity 8000 lux	145	0.16	0.13
	169	0.11	0.16
	196	0.08	0.17
	216	0.29	0.16
	223	0.22	0.11

Acknowledgments

We wish to thank Miss G.J.J. Holland, Mr. E.J. Vlietstra and Mr. C. Hooijer for technical assistance. We also wish to thank Dr. R.J.J.Ch. Lousberg for discussing the manuscript.

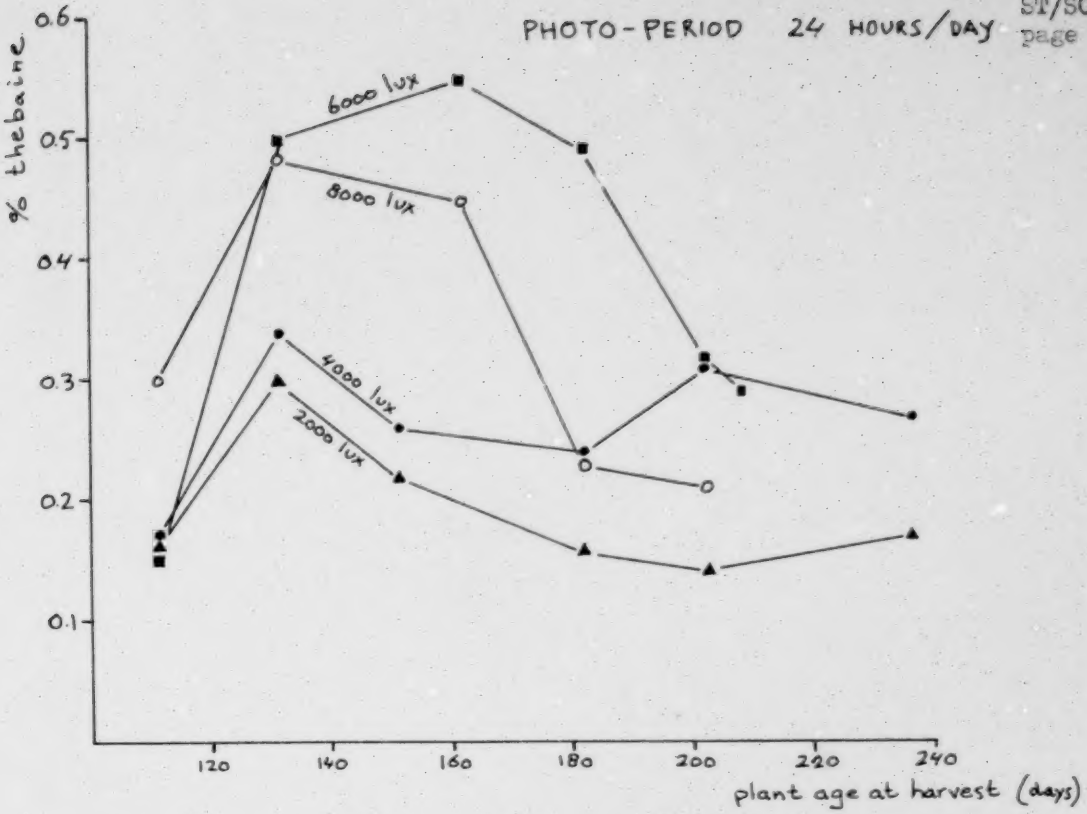
PHOTO-PERIOD 24 HOURS/DAY
% thebaine
0.6
0.5
0.4
0.3
0.2
0.1
6000 lux
8000 lux
4000 lux
2000 lux
120
140
160
180
200
220
240
plant age at harvest (days)

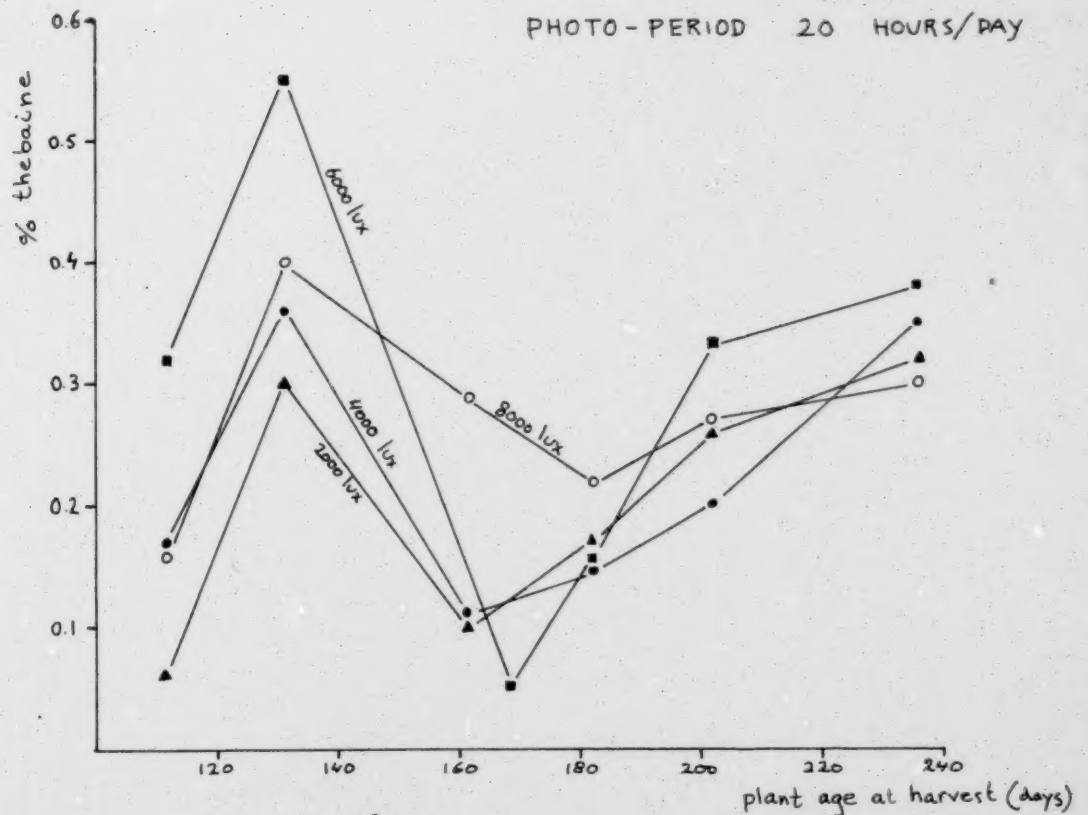
PHOTO-PERIOD 20 HOURS/DAY
% thebaine
0.6
0.5
0.4
0.3
0.2
0.1
6000 lux
8000 lux
4000 lux
2000 lux
120
140
160
180
200
220
240
plant age at harvest (days)

figure 1

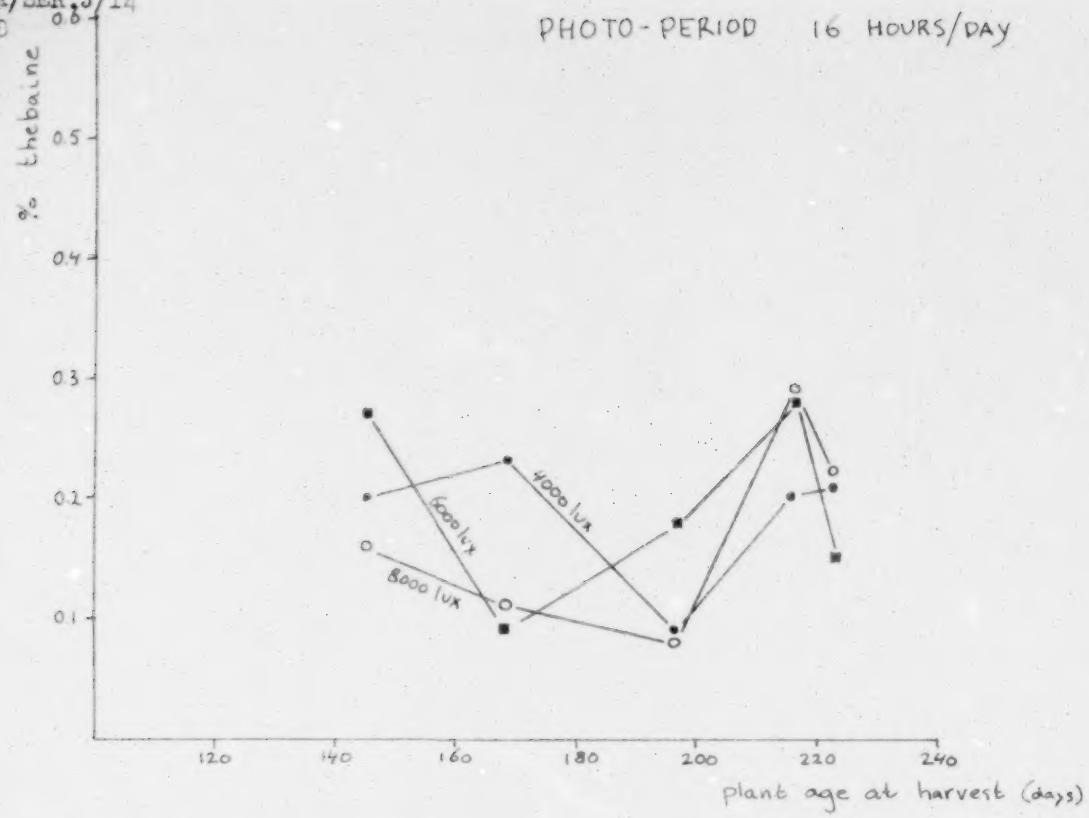
PHOTO-PERIOD 16 HOURS/DAY
% thebaine
0.6
0.5
0.4
0.3
0.2
0.1
6000 lux
4000 lux
8000 lux
120
140
160
180
200
220
240
plant age at harvest (days)

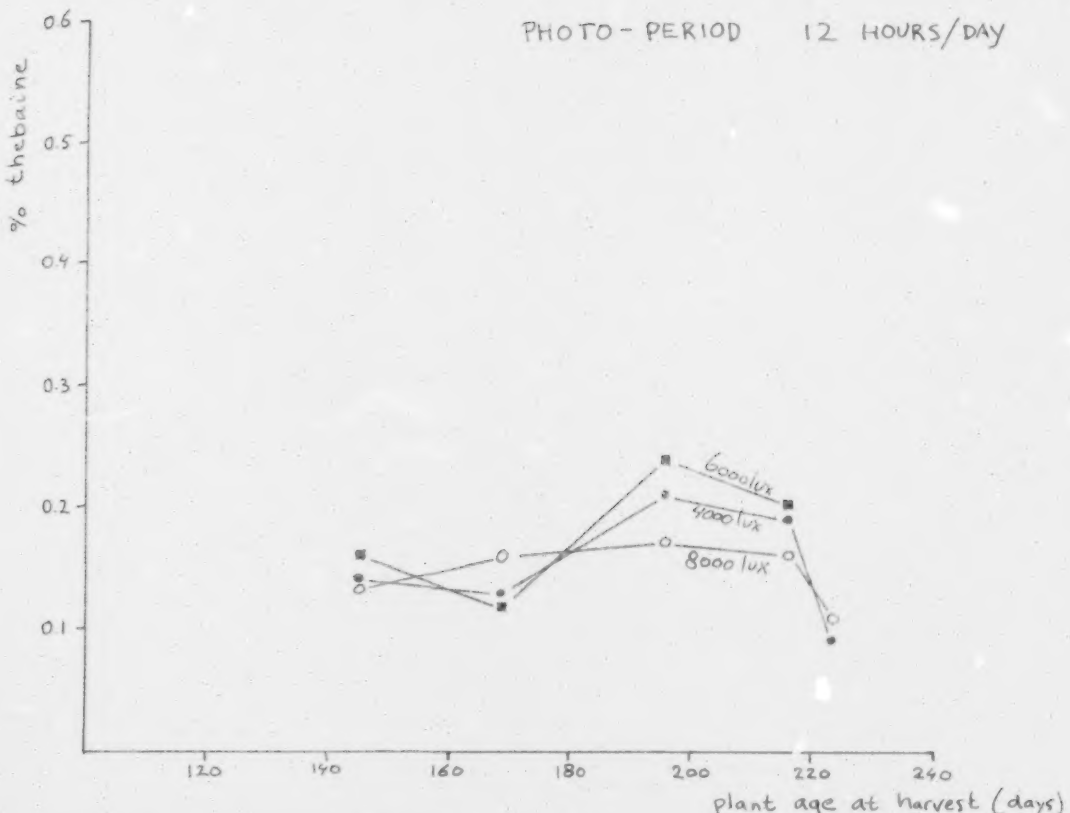
PHOTO-PERIOD 12 HOURS/DAY
% thebaine
0.6
0.5
0.4
0.3
0.2
0.1
6000 lux
4000 lux
8000 lux
120
140
160
180
200
220
240
plant age at harvest (days)

figure 2

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2[illegible] October 1974

Original : ENGLISH

Division of Narcotic Drugs

SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM

No. 15

Report of the Third Working Group on Papaver bracteatum [1]

Beltsville, Maryland, United States of America

7 - 11 October 1974

1/ This meeting was held with the financial support of the United Nations Fund for Drug Abuse Control.

GE. 74-10622

REPORT OF THE THIRD WORKING GROUP ON PAPAVER BRACTEATUM

1. During recent years, considerable interest has been expressed in Papaver bracteatum as a possible source of thebaine and thus, by conversion, of codeine[2]. A collaborative research project on this poppy was initiated by the United Nations Narcotics Laboratory and investigations are being carried out in many countries. Working Groups of experts on Papaver bracteatum were held in Geneva[3] (December 1972) and in Teheran[4] (September 1973).

2. The third Working Group of experts[5] was convened in Beltsville, Maryland, United States of America, from 7 to 11 October 1974, with the financial support of the United Nations Fund for Drug Abuse Control. Facilities for the meeting were provided by the United States Department of Agriculture, through the courtesy of Mr. T.W. Edminster, Administrator of the Agricultural Research Service, United States Department of Agriculture.

3. The meeting was opened by Mr. L.F. Cavanaugh[6] of the United States Department of State who welcomed the Group and stressed the importance of the research being carried out on Papaver bracteatum.

4. Dr. Q. Jones was elected Chairman by acclamation. Dr. O.J. Braenden represented the United Nations and Miss E.S. Lumsden was the Executive Secretary of the Working Group.

2/ Codeine is the most widely used anti-tussive and it is also extensively used as a mild analgesic. It is recognized that there are shortages in the raw materials for the production of codeine, which is mostly derived at present, by the process of methylation, from the morphine obtained from the opium poppy - about 90% of the legally produced morphine being converted into codeine.

3/ See report, document ST/SOA/SER.J/1.

4/ See report, document ST/SOA/SER.J/2.

5/ The list of participants and observers is given in Annex I.

6/ The text of the opening statement is given in Annex II.

5. The Working Group paid tribute to the memory of the late Professor Celâl Tariman of the University of Ankara, who died in an automobile accident in January 1974 and who had participated in the first two Working Groups on Papaver bracteatum.

6. The list of suggested aspects for discussion[7] that had been prepared by the Secretariat, was adopted.

7. It was noted that considerable progress had been made since the initiation of the research, particularly since the second Working Group.

8. It was reported that intensive field study combined with cytological and chemical analysis of Papaver section Oxytona[8] had resulted in the re-evaluation of species in the section[9]. These biosystematic studies showed that P. bracteatum is relatively uniform in important taxonomic characters and is identifiable by its robust habit, usually several conspicuous floral bracts, dark red, blotched petals[10] and, before flowering, by the erect oblong bud with short, appressed, broad-based bristles on the calyx. The chromosome number of all the individuals examined was 2n = 14. Stoma length and pollen diameter are also characteristic for P. bracteatum. The principal alkaloid is thebaine. In some populations alpinigenine is present in small quantities.

9. Certain plants of P. bracteatum were found in northern Iran containing mixtures of thebaine and isothebaine or thebaine and some minor alkaloids. These plants were considered to be natural hybrids of P. bracteatum with P. orientale and P. bracteatum with P. pseudo-orientale respectively.

10. The occurrence of P. bracteatum in Iraq, Turkey and the Union of Soviet Socialist Republics has been reported, but the Group felt that further confirmation was needed on the total natural distribution of this poppy.

7/ See Annex III.

8/ P. Goldblatt, (1974) Ann. Missouri bot. Gard., 61, 264-269.

9/ P. bracteatum, P. orientale and P. pseudo-orientale. A comparison of the characteristics of these three species is given in Annex IV.

10/ The petals turn dark purple on drying.

11. Research was carried out on the possible correlation between growth characteristics and thebaine content. Various parameters were studied including germination conditions, vigour of seedlings, tendency to flower during first year of growth, colour of foliage, number of capsules per plant, size and shape of capsules and colour of pollen. It seemed that there was no correlation between the thebaine content and any characteristic studied.

12. Studies have shown that maximum germination occurred with constant temperature of 10-25°C; 15°C appeared best for general testing.

13. Seedlings grew well in clay, clay-loam and loam soils but poorly in sandy soils or in soils high in organic matter. Seedlings emerged well from soil depths of 0.5 cm or less. Mulching is useful in preventing desiccation damage to which seedlings are prone. With adequate irrigation, the type of seed bed has little effect on seedling establishment.

14. Investigations were carried out to determine the effect of soil pH on germination and seedling establishment. Good results were obtained for germination and seedling growth from pH 5.1 - pH 10 [11]. The pH of the soil is relatively unimportant within this range, but the salts affecting pH are very important. It was noted that the addition of sodium salts seemed to inhibit germination.

15. Direct seeding in the field was successful. Germination continued in field plantings throughout the growing season. Roots of direct-seeded plants grew deeper than 60 cm during the first growing season. Transplanted plants 4 weeks or older were easily established with plug-plantings and were slightly larger than direct-seeded plants at the end of the first growing season.

16. Considerable information has now been developed on vegetative reproduction of P. bracteatum. It was felt that because of the ease with which direct seeding and seedling transplantation in the field can be accomplished and the apparent uniformity of high thebaine yields in plants derived from seed, vegetative propagation would not be necessary in commercial growing of this plant.

11/ The Group realized that soils of pH 10 were rarely, if ever, found in nature.

17. The irrigation requirements of P. bracteatum vary with the type of soil, amount of wind and prevailing temperatures. However it was noted that after the plants were established the water requirements were low compared with other crop plants since P. bracteatum derives moisture from deeper soil layers.

18. Experiments on self-pollination had been successful in some areas and viable seed had been obtained by bagging the opening bud with a paper bag, removing it as soon as the petals fall. However, other investigations of this nature had not been successful.

19. Pest control was a problem - particularly in connexion with weeds. Asulam[12/] can be an effective herbicide for controlling weeds in P. somniferum. Some studies show that it can also be effective in the case of P. bracteatum (except for chenopods). A promising new herbicide, glyphosate, is being tested for weed control in P. bracteatum. Further research is needed to evaluate a wide range of herbicides under many different conditions. The effects of herbicides on the alkaloid profile of P. bracteatum will have to be determined.

20. During intensive experimental cultivation it has been found that, in some localities, P. bracteatum is susceptible to plant diseases and to damage from insects. These problems require further study.

21. Research is needed to determine the most suitable time and methods of harvesting. It was suggested that harvesting at the time of maximum thebaine content of the capsules may not necessarily be the most economical. It may be economically more feasible to harvest after full seed maturity avoiding drying costs and obtaining seed of higher quality. Research will be carried out to determine which portions of the plant should be harvested and the best methods for doing so. The Group felt that detailed consideration of harvesting should be deferred until a future working group.

12/ methyl sulfanilylcarbamate.

22. During 1974, P. bracteatum was cultivated experimentally in many countries, particularly in Australia, the Democratic Republic of Germany, France, Hungary, India, Iran, Ireland, Israel, Japan, The Netherlands, Norway, Poland, Switzerland, United Kingdom, United States of America and Yugoslavia. In this connexion, general appreciation was expressed for the seeds which had been provided by Professor Lalezari of Teheran University through the United Nations Narcotics Laboratory.

23. The thebaine content in the various organs of P. bracteatum plants grown from Iranian and other seeds in various countries had been determined: roots 0.17 - 0.69%; stems 0.3 - 1.0; leaves 0.099 - 0.45%; petals 0.85 - 1.15%; bracts 2.12%; stigmas 0.55 - 0.75%; capsules 0.30 - 5.8%. Although there was considerable variation from plant to plant and between strains, it was noted that Arya II [13/] material gave consistently higher and more uniform values for the thebaine content in the various plant parts.

24. Variations had been reported in the thebaine content in the plant according to the time of day. It was suggested that these might be due to changes in moisture content and not to changes in the absolute amount of thebaine. In P. bracteatum straw, the thebaine content remains constant for long periods of storage.

13/ At the second Working Group on P. bracteatum considerable interest was expressed in new collections from the Mahabad region in the west of Iran. The capsules of these plants have the highest content of thebaine. The pigment and resin content is low and no other alkaloid was detected in the dry capsules. It was thus possible to obtain pure thebaine relatively easily. In view of the importance of this discovery, the second Working Group felt that it would be desirable to nomenclaturally distinguish collections of the Alborz Mountains from those of the Mahabad region by referring to the former as "Arya I" and to the latter as "Arya II". (See document ST/SOA/SER.J/2.)

25. In considering methods for the determination of thebaine in P. bracteatum, the Group felt that several reliable methods were available. However, to facilitate inter-laboratory comparison, it was desirable that a uniform procedure should, in addition, be used in all laboratories. A Sub-Group, under the chairmanship of Professor J.M. Fairbairn considered this question. The conclusions and recommendations of the Sub-Group are given in Annex V.

26. Reference was made to the use of high pressure liquid chromatography techniques for the determination of thebaine. This had the advantage that a simple extract of the sample could be injected directly into the chromatograph. The method was very rapid and the sample required little handling.

27. Where large numbers of analyses were needed, it was desirable to have a rapid method with sufficient accuracy to classify samples according to their relative rather than their absolute thebaine value.

28. In determining the thebaine content of capsules it would be of value to record the developmental stage (days from flower opening) of the capsules analysed.

29. Further investigations were also necessary on the stability of thebaine during the extraction process and storage.

30. The Group felt that the reference sample of capsule material which had been prepared by the United Nations Narcotics Laboratory had been of great value in comparing methods for the determination of thebaine. It was suggested that reference samples should also be prepared of leaf, stem and root tissue.

31. Further research was needed on the biosynthesis, translocation and metabolism of thebaine. The action of enzymes during biosynthetic and degradation processes should also be studied.

32. It had been reported earlier that thebaine occurs in the seeds of P. bracteatum. This was probably due to the contamination of the seeds by the latex as it had not been possible to confirm the presence of thebaine.

33. On the basis of present knowledge, there seems to be little qualitative difference between the oil obtained from P. bracteatum seeds and that from P. somniferum. Data on the oil content and fatty acid composition of P. bracteatum seeds are given in Annex VI.

34. Remarkable similarities were found in the amino acid content of the seed proteins from these poppies. Of the 30 identified amino acids examined, only one significant difference was noted. Twice the amount of methionine 14/ was found in the case of P. bracteatum than in P. somniferum.

35. The Group felt that further research, including animal feeding tests, should be carried out to determine whether P. bracteatum seed oil and meal were suitable for human and/or animal consumption.

36. In summarizing its discussions, the Working Group recommended that during the coming year special attention should be given to research in the following areas:

- weed control: cultural, chemical;
- detailed studies, in geographic areas of potential commercial production, of light, thermal and other ecological factors for maximizing thebaine production from P. bracteatum. These studies will pay special attention to such characteristics as first season flowering, non-shattering capsules and water-resistant capsules;
- in addition to the reference sample of capsule material which has been provided to participants, similar reference samples should be prepared of root, stem and leaf tissue for comparing results obtained by various analytical methods;
- rough screening method for thebaine determination;
- biosynthesis, translocation and metabolism of thebaine;
- disease control: natural resistance, cultural methods, pesticides;

14/ This is of special significance because plant proteins are usually deficient in this essential amino acid.

- what to harvest, when and how for best economic returns;
- selection and breeding for plant improvement;
- seed oil and protein evaluation for food and feed;
- influence of precursors of plant growth regulators (hormonal type) and trace elements upon alkaloidal patterns and content of the plant;
- selective field collecting of additional P. bracteatum germ plasm.

37. Recognizing the importance of the role of the United Nations Laboratory in the research on P. bracteatum, the Group recommended that it continue its activities in the coordination of research and the exchange of information. It was also recommended that close contact should be maintained between the U.N. Laboratory and scientists collaborating in the research.

38. The Group considered that it would be desirable to have a further meeting in 1975. General appreciation was expressed for the invitation of Mr. J. Morice, Director of the Station d'Amélioration des Plantes, Rennes, to hold the fourth Working Group on Papaver bracteatum in France.

ANNEX I

THIRD WORKING GROUP ON PAPAVER BRACTEATUM

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ANNEX II

THIRD WORKING GROUP ON PAPAVER BRACTEATUM

Opening statement made by the
Representative of the United States Department of State,
Mr. L.W. Cavanaugh

It is an honour for me, on behalf of the Department of State, to welcome you all to this meeting on *Papaver bracteatum*.

We believe this gathering is an especially significant one, because a good deal of the credit for the current interest in, and promise of *Papaver bracteatum*, as an alternate source of raw material for codeine, belongs to the Group assembled here.

Under the sponsorship of the United Nations Narcotics Laboratory, at least fifteen countries are cooperating in the research needed to evaluate this plant and its useful products. Without this international cooperation we would likely have had fifteen isolated research efforts with information exchanges having to take the time-consuming route of awaiting publication of research results in many different scientific journals, in many different languages. Through the meetings of this Working Group and the rapid publication avenue provided by the United Nations Narcotics Laboratory, the time frame for this information exchange has been drastically shortened. I believe it is obvious that this has helped all concerned in avoiding unproductive lines of research and in focusing resources on critical problem areas.

The result has been a cooperative effort accomplishing, in less than three years, what would have taken several times as long through uncoordinated efforts by the same number of scientists.

We congratulate the United Nations Narcotics Laboratory for its role as a catalyst in this programme and we wish to express our appreciation to each of you for your contributions to advancing the world community's knowledge of *Papaver bracteatum*.

Please accept our very best wishes for a successful meeting.

ANNEX III

THIRD WORKING GROUP ON PAPAVER BRACTEATUM

Suggested aspects for discussion

Review of present knowledge of Papaver bracteatum (with special reference to research carried out during 1974) and preparation of guidelines for future research. Consideration of the following broad categories:

- agronomy of P. bracteatum;
- chemistry of P. bracteatum;
- utilization of P. bracteatum.

ANNEX IV

Comparison of characteristics of Papaver bracteatum, Papaver pseudo-orientale and Papaver orientale */

Papaver bracteatum	Papaver pseudo-orientale	Papaver orientale
Robust plant of relatively dry slopes, 1,500 to 2,500 m elevation	Robust plants along streambanks, seepage or alluvial areas 1,500 to 2,000 m	Slender plants; of moist or alpine slopes, 1,800 to 3,000 m
Stem 60-120 cm tall, appressed hispid above (spreading in lasiothrix); leaves to 45 cm long, pinnate to pinnatisect, dentate to bidentate	Stem to 80 cm long, somewhat intermediate in pubescence, leaves to 45 cm long, pinnatisect, dentate	Stem 40-100 cm tall, spreading-hispid-villose below; appressed above; leaves to 35 cm long, lacerate to shallowly pinnatisect, dentate
Cauline leaves 5-7, uppermost on upper third of stem	Cauline leaves 5-6, uppermost on upper third of stem	Cauline leaves 2-3, rarely above the middle of stem
Stomatal pores 20-32 microns long	Stomatal pores 43-60 microns long	Stomatal pores 32-45 microns long
Flowers 1-20, the buds not drooping, oblong to obovate, the sides nearly parallel, the apex truncate; peduncle 3-4 mm thick	Flowers 1-10; the buds erect or drooping, obovate, as in orientale	Flowers 1-4, buds drooping, rounded at top, the sides not parallel, peduncle 2-3 mm thick
Bracts (0)-5-8, mostly calyx like, 1-5 cm long, entire to serrate or lobate	Bracts 0-4, entire to dentate	Bracts usually absent; peduncles free of leaves for some distance

*/ This summary was compiled by James A. Duke, Plant Taxonomy Laboratory, Plant Genetics and Germplasm Institute, Agricultural Research Service, United States Department of Agriculture.

Papaver bracteatum	Papaver pseudo-orientale	Papaver orientale
Sepal hairs appressed, conical, somewhat 3-cornered at the base	Sepal hairs slender and spreading as in orientale	Sepal hairs spreading, cylindrical, round at the base
Petals usually 6, to 10 cm long; sepals 3	Petals 4 or 6, sepals 2 to 3	Petals usually 4, to 9 cm long, sepals 2
Petals scarlet, with longitudinal black blotches near base, drying deep wine purple	Petals orange-scarlet, with rectangular black blotches (sometimes unmarked), drying purplish to pinkish brown	Petals pale orange without maculations, drying pale pinkish brown
Filaments to 1.5 cm long; anthers linear, purple, 2.8-5.5 mm long; pollen 24-27 microns in diameter	Filaments to 1.3 cm long; anthers linear, purple, 1.7-3.5 mm long; pollen 27-31 microns in diameter	Filaments to 1 cm long; anthers oblong, yellow to violet, 1.8-3 mm long; pollen 26 to 29 microns in diameter
Capsules 0.5-3 cm long, 1-4 cm broad, the tops convex, flat, or concave, not changing with age; rays 10-24, the teeth acute	Capsules 0.5-2.5 cm long, the tops flat; rays 9-19	Capsules 0.5-2.0 cm long, starting convex, becoming flat with age; rays 8-15, the teeth obtuse
Rosette seedling leaves more dissected, serrate, hairy, less glaucous	Rosette seedling leaves very much like bracteatum, hairy but not glaucous	Rosette seedling leaves less dissected, serrate and hairy, more glaucous
Haploid chromosome number 7	Haploid chromosome number 21	Haploid chromosome number 14
Thebaine present, regardless of minor alkaloidal variance; alpinigenine frequent	Isothebaine, traces of thebaine and other alkaloids present	Thebaine usually if not always absent, oripavine often dominant, with isothebaine secondary

ANNEX V

Report of the Sub-Group on Methods for the Determination of Thebaine

(Chairman - Professor J.W. Fairbairn; Secretary - Dr. P.G. Vincent)

The objectives of the Sub-Group were to provide general guidelines for the analyses of tissue from P. bracteatum that would be compatible with the laboratory facilities of all*/participating members. Criteria for the guidelines included that the method be simple, accurate and cover a wide range of plant material and quantities of thebaine.

1. Drying of Plant Tissue:

It was recommended that plant tissue be oven dried at 60°C to a constant weight as soon as possible following collection. Several members took exception to this procedure noting that thebaine may be lost using this protocol and that freeze-drying of samples would possibly be better. However, not all members had access to freeze-drying equipment. It was suggested that tissue be cut-up to facilitate oven drying, especially root tissue.

2. Particle Size:

It was recommended that samples be powdered so that all the sample passes through a 0.5 mm mesh and is thoroughly mixed immediately before weighing for analysis. Weight correction due to hygroscopic water absorption should be made by heating a small portion to 105°C and re-weighing at the time of analysis.

3. Sample Size for Analysis:

It was recommended that 0.1 to 0.5 grams of sample be used in analyses.

4. Extraction Solvent:

Acetic acid (5%) was suggested as the extraction solvent but would be considered tentative until fully evaluated by the members.

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*/ Not all members were equipped to participate in a total refereed quantitative study.

5. Procedure:

(a) Samples should be agitated for 30 minutes in 25 ml of acetic acid (5%) solution. The Sub-Group recommends that shorter or longer agitating times be evaluated for efficacy of extraction technique.

(b) Filter eluent through a sintered glass funnel and repeat extraction two further times. Combine the filtrates.

(c) Add 25 ml of $CHCl_3$ and adjust the pH of the aqueous phase to pH 9.5-10 with concentrated NH_4OH with agitation. Separate $CHCl_3$ phases.

(d) Repeat extraction of the aqueous phase with one 25 ml portion of $CHCl_3$ followed by two 15 ml portions of the same solvent.

(e) Combine the $CHCl_3$ phases and evaporate to dryness preferably in vacuo at 40°C.

(f) To the flask containing the residue, add a known volume of ethanol containing an appropriate quantity of internal standard. Suggested internal standards include tetrahydrothebaine, cholesterol acetate, antipyrine and laudanosine. Use of one or more internal standards may be appropriate for certain analytical applications.

6. Gas-liquid Chromatography:

The Sub-Group recommended that deactivated glass columns or glass-lined steel columns be used to avoid decomposition of thebaine (via metal catalysis). It was suggested that the columns be packed with 100/120 mesh Gas Chrom Q coated with 2-3% OV-17 liquid phase or their equivalents. Suggested operating temperatures should range between 260-280°C with either nitrogen or helium as carrier gas.

7. Results:

Results should be calculated on the basis of moisture-free content of plant tissue after drying at 105°C. */

Results from the above procedure should be evaluated using plant material designated UNB-17 and other reference samples and data forwarded to the United Nations Narcotics Laboratory by the end of February 1975.

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*/ Thebaine standardization: check the purity of the standard thebaine by determining the $E^{1\%}_{1\,cm}$ in 0.1N HCl at 285 nm or at the λ max.

ANNEX VI

Analysis of seed samples of P. bracteatum

The oil content was estimated by NMR and the fatty acid composition by gas chromatography.

	UNB-4	UNB-18	UNB-21
oil content	40.2%	36.2%	43.3%
fatty acid composition			
palmitic acid	8.0%	7.1%	7.9%
palmito-oleic acid	0.3%	0.3%	0.5%
stearic acid	2.5%	2.5%	2.5%
oleic acid	12.5%	8.7%	12.0%
linolic acid	76.3%	80.8%	76.8%
arachidic acid	0.5%	0.6%	0.7%

Data provided by Dr. J. Krzymanski, Institute of Plant Breeding and Acclimatization, Poznań, Poland.

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SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM [1]

No. 16

DETERMINATION OF THEBAINE IN PAPAVER BRACTEATUM [2]

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1/ General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

2/ In the report of the Second Working Group on Papaver bracteatum (document ST/SOA/SER.J/2), it was recommended that methods for the determination of thebaine in Papaver bracteatum should be compared and evaluated; to achieve this, it was decided that participants in the Working Group would forward their methods for publication in this series so that these could be tried out in the various laboratories.

GE. 74-12056

In the course of our work on the extraction of poppy capsules, we have developed analytical methods for the quantitative determination of opium alkaloids, including thebaine.

These methods are based on preliminary separation of the alkaloids by thin-layer chromatography, after which the alkaloids are traced under shortwave UV-light. After removal from the TLC-plate, the alkaloids are eluted from the absorbent. An aliquot of the eluate is treated with a reagent and the absorbance of the resulting solution is measured.

In the case of thebaine, the separation is carried out on silica gel. After elution with methanol, the method of Sakurai[3/] is used for thebaine determination.

Equipment

The sample solutions are applied on the TLC-plate by means of a 50 microlitre Hamilton pipette. For the detection of the alkaloid bands, a Camag shortwave UV-lamp (254 nm) is used.

The alkaloid bands are scraped off by means of a bent spatula. The silica gel is transferred into reagent tubes with a vacuum zone collector which consists of a G-3 glass filter (Ø 1 cm) provided on the upper side with a stopcock. The stopcock is connected with a vacuum pump and the silica gel is collected on the under side of the filter plate. The silica gel is transferred to reagent tubes by closing the stopcock and gently tapping the filter against the inner wall of the tube. A Zeiss Spectrophotometer type PM Q II is used for the absorption measurements.

Chemicals and reagents

All chemicals are of reagent grade. The solutions of 4-aminoantipyrine and potassium ferricyanide are prepared daily. The thebaine content of the standard thebaine HCl solution is determined by non-aqueous titration with perchloric acid. The standard solution of thebaine HCl, containing 0.6% (w/v) thebaine calculated as the base, is stored at 5°C.

3/ H. Sakurai, J. Pharm. Soc. Japan, 80, 909 (1960)

Extraction methods

Ground poppy capsules are mixed with 1.6 times v/w ammonia 0.35 N. After standing for 30 minutes, the mixture is extracted with chloroform. The residue from the chloroform extract is taken up in dilute acetic acid. For the analysis of Papaver bracteatum, the acetic acid solution is used as the starting solution for the chromatographic separation. In the case of a low thebaine content and extraction of a relatively large quantity of plant material, the acid solution is extracted at pH 11-13 with toluene. The toluene extract is shaken with dilute acetic acid and this aqueous extract is used for the thebaine analysis.

An alternative method is a soxhlet extraction of the capsule-ammonia mixture with toluene for 10 hours. In this case, the toluene extract is evaporated and the residue treated with ethanol. The ethanol solution is filtered through a glass filter and the filtrate is used for the thebaine analysis.

Description of the analytical method

Extraction of Papaver bracteatum capsules

One gram of ground capsules is treated with 1.6 ml ammonia 0.35 N. After leaving for 30 minutes, the mixture is shaken for 1 hour with 10 ml chloroform. The material is filtered and the extraction is repeated twice with fresh portions of 10 ml chloroform. The extracted papaver material is discarded.

The combined chloroform extracts are evaporated under reduced pressure at a moderate temperature. The still warm residue is dissolved in 3 ml aqueous acetic acid 0.5 N. The solution is quantitatively transferred to a 5 ml volumetric flask by the addition of two portions of about 1 ml acetic acid 0.5 N. The volumetric flask is filled to the mark and the extract is homogenized.

Chromatography

On a silica gel plate GF_{254} (20 x 20 cm) lines are drawn with a pencil making 5 rectangles of 11.5 x 3.8 cm. At the sides however, strips with a breadth of 0.5 cm are drawn which will not be used.

The solutions are applied 1.5 cm from the lower edge of the plate forming a band 3.4 cm wide. Formation of the band can be effected by the application of small drops half overlapping each other. Widening of the band can be prevented by drying with a gentle stream of nitrogen during application. In this way, a band is obtained not broader than 7 mm. If the rectangles are numbered 1 to 5, from left to right, the solutions are applied as follows:

1 : no solution (blank)

2 and 3: 40 microlitres sample solution

4 and 5: 40 microlitres of thebaine standard 0.6%

Note: For the analysis of only one sample, two plates are used to ensure having a sufficient number of blanks and standards.

The chromatograms are developed with a mixture of cyclohexane-diethylamine (9 + 1) and dried at room temperature for 30 minutes. The thebaine bands are marked under shortwave UV and scraped off and collected with the vacuum zone collector. In rectangle 1 a band is drawn with about the same width as the thebaine bands and this is scraped off.

The silica gel is transferred to round bottomed centrifuge tubes and 3 ml methanol is added. The tubes are closed with plastic stoppers and, after careful mixing, they are placed in the dark until the next day.

Colorimetry

The tubes are shaken again and then centrifuged. From the supernatant fluid, 2 ml is carefully pipetted into round bottomed 15 ml centrifuge tubes.

The tubes are placed in a boiling waterbath until the methanol has completely evaporated. 2 ml HCl 3.5% (w/v) is added to the residue in the tubes. After the residues have dissolved, the tubes are closed with glass beads, placed in a boiling waterbath for exactly 25 minutes and then cooled under a stream of tap water. 2 ml 4-amino antipyrine solution 0.375% (w/v)

and 1 ml potassium ferricyanide solution 0.6% (w/v) are added and, after exactly 1 minute, 2 ml ammonia 10% (w/v). The tubes are closed again with the glass beads and heated in a boiling water bath for exactly 5 minutes. After cooling, 1 ml ethanol is added. The tubes are closed with plastic stoppers and the contents are mixed well. The extinction of the resulting solutions is measured at 435 nm against water.

Calculation

The thebaine content of the original sample is calculated as follows:

$$\frac{0{,}6\ V\ (E_s - E_b)}{W\ (E_{st} - E_b)} = \quad \% \text{ thebaine}$$

Where: E_s = extinction measured for the sample
E_{st} = extinction measured for the standard
E_b = extinction of the blank
V = the total volume of the sample solution in ml
W = sample weight expressed in grams.

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SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM 1/

No. 17

LIQUID CHROMATOGRAPHIC ASSAY OF THEBAINE IN PAPAVER BRACTEATUM 2/

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1/ General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

2/ In the report of the Second Working Group on Papaver bracteatum (document ST/SOA/SER.J/2), it was recommended that methods for the determination of thebaine in Papaver bracteatum should be compared and evaluated; to achieve this, it was decided that participants in the Working Group would forward their methods for publication in this series so that these could be tried out in the various laboratories.

3/ This paper was submitted by Dr. E.F. Schoenewaldt, Manager, Process Research, Merck and Co. Inc.

GE. 74-12275

Introduction

A simple assay procedure featuring a methanolic-ammonia extraction and a high pressure liquid chromatographic separation (cation exchange) is described for thebaine in Papaver bracteatum. The results of this procedure for three P. bracteatum samples obtained from Dr. Phillip Vincent of the United States Department of Agriculture are shown in Table I. These samples were assayed as received. The effects of sample particle size distribution, extraction efficiency and stability of thebaine on the precision of the assay are discussed.

Experimental

A. Apparatus

1. Liquid chromatograph - Du Pont Model 840.
2. 25 microliter syringe - Hamilton 702.
3. 15 milliliter centrifuge tubes fitted with polyethylene caps.
4. Ultrasonic bath.

B. Chemicals

1. Boric acid.
2. 50% Sodium hydroxide.
3. Potassium nitrate.
4. Acetonitrile - spectroquality.
5. n-Propanol - spectroquality.
6. Methanol.
7. Ammonium hydroxide.

C. Solutions

1. Extractant - 98/2 (v/v) methanol/ammonium hydroxide.
2. Chromatographic eluent - 0.3M NaOH adjusted to pH 9.5 with boric acid, 0.2M KNO_3, 4% acetonitrile, 1% n-propanol.

   (a) Preparation of 4 liters of the 0.3M NaOH solution:

   50% Sodium hydroxide - 60 ml
   Boric acid - 114 g (approximate)
   Distilled water - 4000ml

(b) Preparation of 4 liters of the chromatographic eluent:

3800 ml - Solution from Step (a)
80.9 g - Potassium nitrate
160 ml - Acetonitrile
40 ml - n-Propanol

(c) Degas the solution prepared in Step (b) by bubbling helium through the solution for 5-10 minutes.

D. Chromatographic Conditions

Column - 1 m x 2.1 mm (id) strong cation exchange, Du Pont SCX.

Eluent - 0.03M NaOH adjusted to pH 9.5 with boric acid, 0.2 M KNO_3, 4% acetonitrile, 1% n-propanol.

Pressure - 1200 psig.

Flow - 0.8 ml/min.

Temperature - ambient.

Detection - UV_{254} nm, attenuation 0.02 AUF.

Chart Speed - 0.2 in/min.

Injection Size - 10.0 microliters.

E. Thebaine Standard - 0.400 mg/ml

Weigh accurately 10 mg of the thebaine reference material into a 25 ml volumetric flask and dilute to the mark with 98/2 (v/v) methanol/ammonia. The standard solution is stable for at least a week if refrigerated.

F. Assay Procedure

1. Weigh accurately 200 mg of the sample into a 15 ml centrifuge tube.
2. Accurately pipette 3 ml of the 98/2 (v/v) methanolic/ammonia extractant into the tube if the sample is roots or shoots and 10 ml if the sample is a capsule.
3. Cap the tube with a polyethylene cap and shake continuously for 20 minutes with occasional ultrasonification.
4. Centrifuge the sample for 5 minutes to clear the liquid layer.
5. Inject duplicate aliquots of exactly 10 microliters of the sample solution (liquid layer - Step 4) into the liquid chromatograph.

6. Inject duplicate aliquots of exactly 10 microliters of the thebaine standard solution into the liquid chromatograph.

7. Calculate the Wt % of thebaine in the sample by the following relationship:

$$\text{Wt \% thebaine} = \frac{A_X \cdot C_S \cdot D \cdot 100}{A_S \cdot W_X}$$

where A_X = Sample thebaine peak area.
A_S = Standard thebaine peak area.
C_S = Concentration of the thebaine standard.
W_X = Weight of the thebaine sample.
D = Milliliters of extractant.

example - Capsule (P. bracteatum, Arya II from Iran, 12/73)

$A_X = 2.77\ in^2$ [peak area = hxw (at 1/2 h)]
$A_S = 2.75\ in^2$
$C_S = 0.507$ mg/ml
$W_X = 178.5$ mg
$D = 10$

$$\text{Wt \% thebaine} = \frac{2.77 \cdot 0.507 \cdot 10 \cdot 100}{2.75 \cdot 178.5} = 2.86$$

Results and Discussion

The results of the thebaine assay for three samples sent to Dr. Schoenewaldt by Dr. Vincent of the USDA are listed in Table I, and their liquid chromatograms are shown in Figures 1, 2 and 3. The USDA assays on the root and shoot samples are reported to be 0.4% and on the capsule to be 4.3%. Our liquid chromatographic assays on these samples are 30-50% lower. Three factors were investigated to account for these discrepancies.

1. Thebaine solvent stability - The assay of a root extract did not significantly change after 5 days of aging the extract at room temperature. The solvent was the 98/2 (v/v) methanolic/ammonia extractant.

2. Extraction efficiency - A root sample was sampled after 20 minutes and 90 minutes of extraction. The assays were not significantly different.

3. Sample particle size distribution - An aliquot (2.9 g) of the capsule sample (ground to 40 mesh) was dry sieved for 20 minutes into four fractions and each fraction then assayed for thebaine. The results of these experiments are listed in Table II. The data clearly show that the 40 mesh sample is inhomogeneous with respect to thebaine content because the smaller particle fractions contained more thebaine than did the bulk sample. The amounts of thebaine obtained from the weighted sum of the four fractions and from the bulk sample were not significantly different (87.3 mg vs 86.0 mg) suggesting that the solvent extraction was complete and that the bulk sample had not yet suffered much particle size segregation (i.e. smaller particles settling to the bottom of the vial). An additional experiment was performed on the 100 mesh fraction material. This material was ground by mortar and pestle and then separated into two fractions with a 100 mesh sieve. The amounts of thebaine obtained from the weighted sum of the two fractions, and the original 100 mesh material were not significantly different as shown in Table III. If the weighted sum assays had been significantly larger than the bulk assays, then one could have assumed that the smaller particle material was extracted more efficiently than the larger particle material. The data do not support this argument. These experiments clearly show that the solvent extraction is complete and suggest that there are fines rich in thebaine which adhere to the larger sample particles. Experiments should be performed to determine the optimum sample size, sieve fraction and sieving time to produce an accurate assay for thebaine in the various parts of the plant.

Conclusion

The liquid chromatographic assay for thebaine is a simple procedure requiring few laboratory steps. However, the precision of the assay and inter-laboratory assay correlation will depend on a well defined sampling procedure. It is apparent from the data in Table II that sampling 40 mesh ground plant material could lead to erroneous results.

Table I

Liquid chromatographic assay of thebaine in various P. bracteatum samples

Sample	Sample weight	Extractant Volume	Wt. % thebaine
Roots (UNB 4)	190.5 mg	3 ml	0.22
Shoots (UNB 4)	173.5 mg	3 ml	0.08
Capsule (Arya II from Iran, 12/73)	178.5 mg	10 ml	2.86

Table II

Thebaine content of various particle size fractions of the P. bracteatum 40 mesh capsule sample

Mesh size	Sieve Wt	Data %	Wt of thebaine	Wt % thebaine
>40 (420μ)	2.90 g	100%	86.0 mg	2.97
<100 (149μ)	1.37 g	47.2	27.3 mg	1.99
100-200 (149μ - 74μ)	.47 g	16.2	18.9 mg	3.40
200-325 (74μ - 44μ)	.80 g	27.6	29.5 mg	3.69
>325 (44μ)	.26 g	9.0	11.6 mg	4.46
		100.0	87.3 mg	

Table III

Thebaine content of the ground 100 mesh fraction of the P. bracteatum capsule sample

Mesh size	Sieve Wt	Data %	Wt of thebaine	Wt % thebaine
> 100	1.22 g	100%	24.2 mg	1.99
> 100	.78 g	64%	11.2 mg	1.44
< 100	.44 g	36%	12.4 mg	2.81
		100%	23.6 mg	

Figure 1. Roots, P. bracteatum

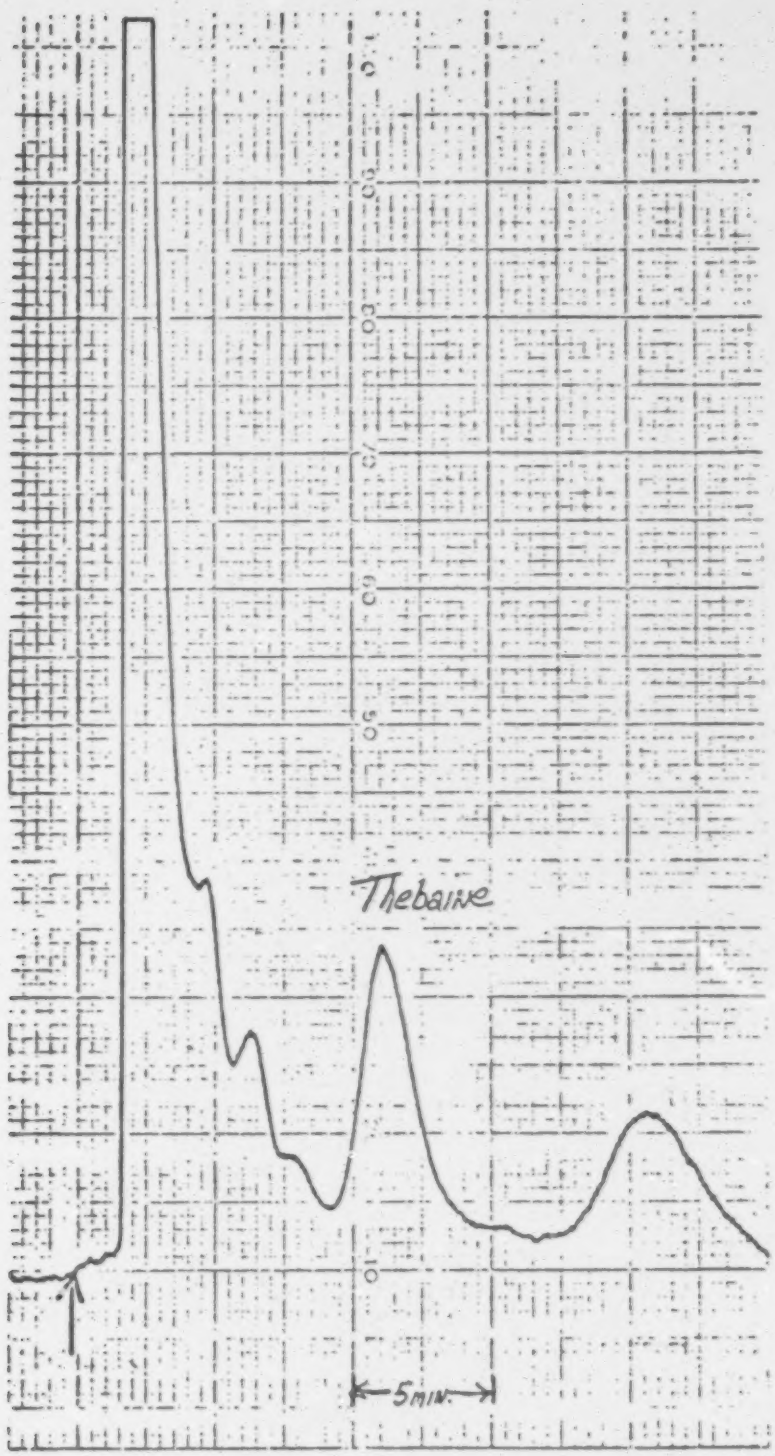


Figure 2. Shoots, P. bracteatum

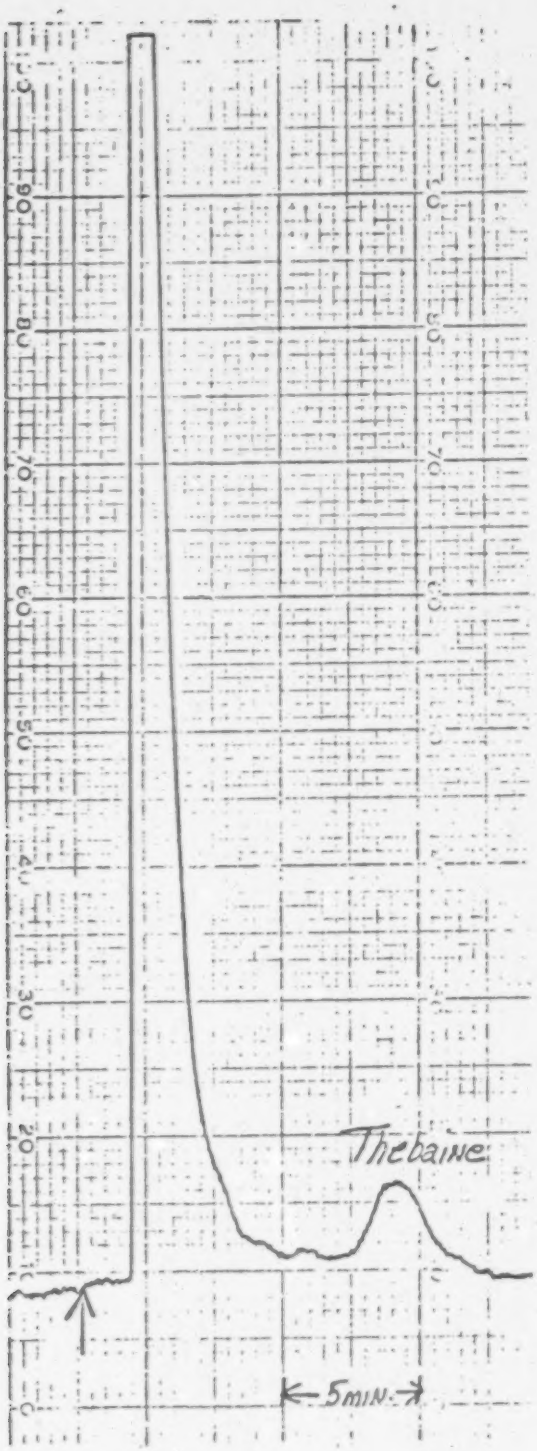
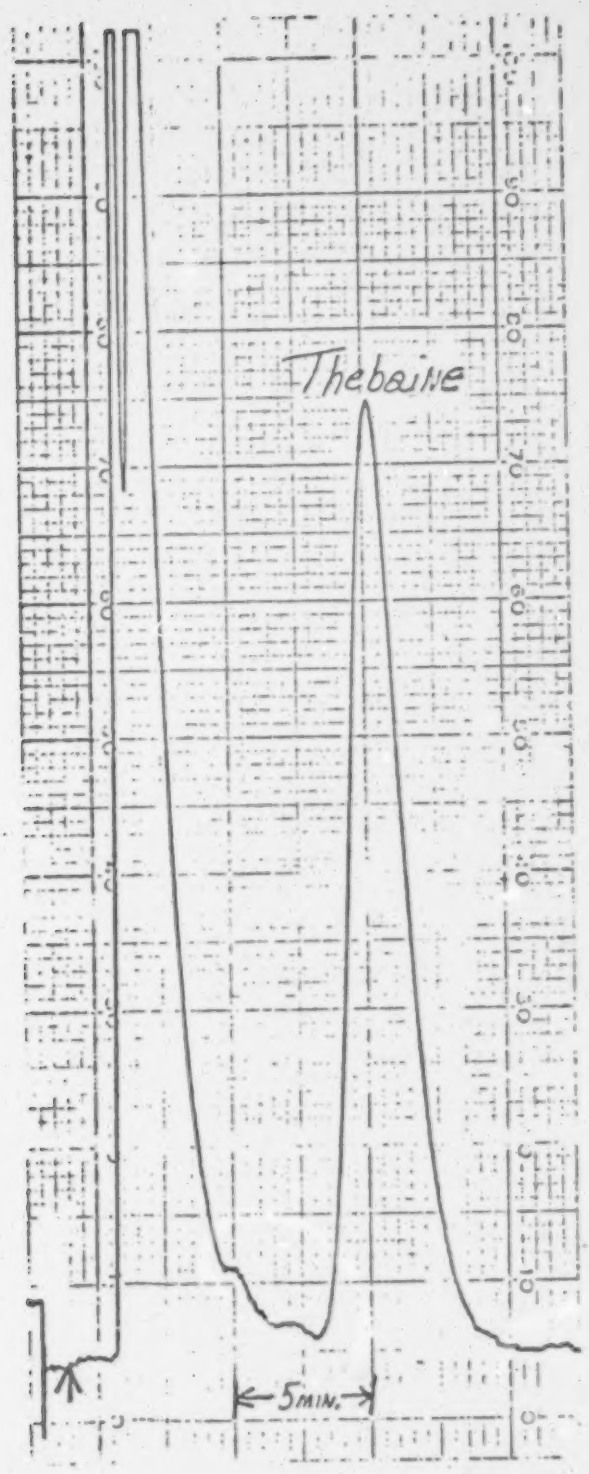


Figure 3. Capsule, P. bracteatum



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SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM 1/

No. 18

DETERMINATION OF THEBAINE IN PAPAVER BRACTEATUM 2/

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1/ General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

2/ In the report of the Second Working Group on Papaver bracteatum (document ST/SOA/SER.J/2), it was recommended that methods for the determination of thebaine in Papaver bracteatum should be compared and evaluated; to achieve this, it was decided that participants in the Working Group would forward their methods for publication in this series so that these could be tried out in the various laboratories.

3/ This paper was submitted by Dr. J.A. Snyder, Market Research Manager, Pharmaceuticals Division, Biochemical Department, E.I. du Pont de Nemours & Co. Inc., Wilmington, Delaware 19898, U.S.A.

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Preparation of plant material prior to assay

Grind the sample with a suitable mill[4] to pass a 40 mesh screen. Dry the ground plant material to constant weight at 60°C in air. Store the dried, ground sample in a desiccator under vacuum.

Preparation of sample residues for chromatography

Transfer a portion of the ground, dried plant material, accurately weighed (refer to Table I), into a stoppered flask. Add 30.0 ml 3.0% (v/v) acetic acid and shake for 20 minutes. Filter through a medium porosity fritted glass funnel with the aid of vacuum, collecting the filtrate in a 125-ml separator. Return the plant material to the flask and extract as before with 20.0 ml 3.0% (v/v) acetic acid. After the second extraction, rinse the funnel with 5 ml 3.0% (v/v) acetic acid, collecting the washings in the separator.

Adjust the pH of the combined extracts and washings to 8.7-9.3 (check with short-range pH paper), using ammonia:ammonium chloride buffer[5]. Typically, 15-20 ml are required to raise the pH to the desired range. Extract with two 25-ml portions of benzene. Shake the separator gently since samples tend to form emulsions during the extraction. If the emulsion does not break on standing, allow the contents of the separator to drain slowly into a second separator. Now the emulsion should have broken sufficiently to allow separation of the benzene phase. Pass the benzene phase through a pledget of benzene-wetted glass wool that has been previously washed with benzene and collect these extracts in a 100-ml round-bottom flask. Rinse the glass wool with a few ml of benzene, adding the rinsings to the extracts. Evaporate the combined extracts and rinsings to dryness on a suitable rotary evaporator with the aid of vacuum[6]. The residue thus obtained is the sample residue.

4/ e.g. Wiley Laboratory Mill, Model PRI. Available from A.H. Thomas Co., Philadelphia, Pa.

5/ pH = 9.5, 2.5M NH_3 and 2.5M NH_4Cl (the buffer is prepared by dissolving 33.5 g NH_4Cl in about 150 ml water, adding 42 ml concentrated ammonia and diluting to 250 ml with water).

6/ e.g. Buchi Rotavapor R with heating bath at 35-40°C. Available from Brinkmen Instruments, Inc., Westbury, New York.

Table I

	For thebaine content less than 1%, use	For thebaine content greater than 1%, use
Dried ground plant material	500 mg	200 mg
Aliquots of thebaine stock standard	3.00, 6.00 & 10.00 ml	6.00, 10.00 & 15.00 ml
Internal standard	2.00 ml	4.00 ml

Preparation of standard residues for chromatography

Transfer 50 mg of thebaine reference standard, accurately weighed, into a 100-ml volumetric flask. Dissolve and bring to volume with 3.0% (v/v) acetic acid. Prepare three standards of varying concentration by pipetting aliquots of this stock solution (refer to Table I) into separate 125-ml separators. Add sufficient 3.0% (v/v) acetic acid to bring the total aqueous volume in each separator to 55 ml.

Proceed as directed in preparation of sample residues for chromatography, beginning with the phrase "Adjust the pH ...". The residues thus obtained are the standard residues.

Internal standard preparation

The internal standard is an n-butanol solution of cholesterol acetate at a concentration of 1.0 mg per ml.

Gas chromatography of standards and samples

Dissolve each residue in an identical volume of internal standard (refer to Table I). Inject the most concentrated standard on a suitable gas chromatograph[7/], equipped with a flame ionization detector at the conditions suggested below, until successive injections give thebaine:internal standard peak area ratios that agree to better than two percent.

7/ e.g. Hewlett-Packard, Model 402. Available from Hewlett-Packard, Paramus, New Jersey.

Column:	4' x 3.5 mm, I.D. glass column with 3.8% OV-17 Gas Chrom Q, 100-120 mesh
Oven:	250°C
Flash Heater:	280°C
Detector:	290°C
He (carrier) Flow Rate:	about 75 ml/minute
H_2 Flow Rate:	about 30 ml/minute
Air Flow Rate:	about 350 ml/minute
Chart Speed:	1/4 inch/minute
Approximate Retention:	4 minutes: thebaine 11 minutes: cholesterol acetate.

Typically it is found that a 2 µl injection will give the desired response and that two to four injections are necessary to achieve the desired precision. It is sometimes found that data from the first few injections should be omitted because the column response is not stabilized. Inject each remaining standard twice and then each sample once in turn. Chromatograms of some plant material show extra peaks after the internal standard. Therefore, injections should be made at least 20 minutes apart for the first injection of all samples. Each sample is to be injected twice, or until a 2 percent coefficient of variation is obtained for the peak area ratio of that sample. After every eight sample injections (or more frequently if necessary), alternately inject a standard. After all samples, make a final injection of each standard.

Calculation of thebaine content

Compute a mean thebaine:internal standard peak area ratio for each standard and sample. Make a linear plot from the standard data of the amount of thebaine (mg/round-bottom flask) versus mean peak area ratio. Interpolate the weight (mg) of thebaine in each plant sample from the mean peak area ratio found for the sample, and compute the thebaine content by:

$$\text{Percent thebaine in plant sample} = \frac{\text{Thebaine found (mg)}}{\text{Anhydrous sample Wt. (mg)}} \times 100$$

Analytical data on P. bracteatum samples

Plant material	Number	% thebaine (anhydrous)	Capsule weight (g)
UNB-17 Reference sample (powder)	Run 1	3.40	-
	2	3.39	-
	3	3.43	-
	4	3.41	-
	Mean	3.41	-
Arya II capsules (Prof. Lalezari)	1	3.56	2.23
	2	2.60	1.27
	3	3.96	2.54
	4	--	1.31
	5	5.84	1.95
	6	--	1.88
	7	3.42	1.89
	8	3.52	1.33
	9 10	2.87 */	2.30
	11	5.40	3.03
	12	--	1.28
	13	--	1.30
	14	2.53	1.26
	15	--	1.10
	16	--	1.49
	17	4.07	1.42
	18	2.69	2.37
	19	--	1.94
	Mean	3.53	1.80

*/ Capsules 9 and 10 were combined after weighing and analyzed as a composite sample.

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SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM [1]

No. 19

Identification of Papaver bracteatum and other species of Papaver section Oxytona from leaf epidermis impressions [2]

by: Peter Goldblatt, [3]

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1/ General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

2/ This work was supported by Grant 12-14-100-9963(34) from the Agricultural Research Service of the United States Department of Agriculture.

3/ This paper was submitted by Dr. Quentin Jones, Chairman of the Third Working Group on Papaver bracteatum.

GE. 75-4326

Although section Oxytona of Papaver, to which P. bracteatum is assigned, is itself distinct in the genus, species identification within the section is difficult, and has been up till now possible only from flowering or fruiting material. With the increasing importance of P. bracteatum as a potential crop plant and experimental subject, identification of seedling material is necessary in preventing considerable waste of time and effort in growing unwanted species.

As a result of a taxonomic study supported by the Agricultural Research Service of the United States Department of Agriculture on section Oxytona, which has included cytological data, the systematics of the section is now much better understood. The results of this study (Goldblatt 1974) have indicated the existence of three distinct species in the section, each chemically and cytologically, as well as morphologically, quite distinct. The three species are Papaver orientale L. (2n=28); P. bracteatum Lindl. (2n=14) and P. pseudo-orientale (Fedde) Medw. (2n=42). Of the three, only P. bracteatum contains significant amounts of thebaine, either as the only alkaloid or in combination with small amounts of alpenigenine.

The differences in ploidy level among the three species are primarily what has made their distinction possible from seedling stage onward. The seedlings are in fact also morphologically distinct although not easy to recognize except by a very trained eye, and identification on external morphology of the seedlings is not practical. However, species can be determined not only by direct cytological examination but also by the size of cells in the leaf. The feature most readily observed and measured is the stoma, the pore found in the epidermis of plants and in the leaves of Papaver, only on the lower surface.

Method

To examine the epidermis rapidly a cellophane impression is made of the leaf and examined under a compound microscope. There are several methods for producing epidermal impressions, but the most suitable for Papaver has been that described by Payne (1970). Strips of cellulose acetate film (0.0025-0.005 inches thick) are used, cut into pieces about 2 inches long and wide enough to cover a small, detached portion of leaf. The leaf portion is first placed lower surface uppermost on a

glass slide, flooded with acetone, and then the cellulose acetate strip is placed over the leaf and also moistened with acetone. One end of the film should be secured with Scotch tape to the slide.

Only when the acetone has quite evaporated should the leaf be removed, and the other end of the strip should be taped to the slide, making the resulting impression lie as flat as possible but taking care not to stretch it.

Results

Examination of many plants of numerous wild collections from Iran and Turkey has shown that epidermal cell and stoma size are directly related to chromosome number. Thus, Papaver bracteatum has the smallest cells and stomata while P. pseudo-orientale has the largest. Since the stomata are of a regular shape in contrast to the epidermal cells surrounding them which have very sinuous margins, the stomata are most suitable for measurement and comparison. While there is some variation in size between individual stomata, measurement of ± 25 stomata and averaging the total has invariably resulted in the placing of seedlings in their correct species.

Species	Ploidy	Range & mean stoma length	
P. bracteatum	2n	20-32	26
P. orientale	4n	32-45	36
P. pseudo-orientale	6n	43-60	50

Table 1. Range and mean stoma length in Papaver section Oxytona.

Conclusion

Seedlings of the three species recognized in Papaver section Oxytona differ in their ploidy. Papaver bracteatum is the only diploid. Plants known to belong to this section can accordingly be identified at all stages of their development from seedling onwards either by direct cytological examination and determination of chromosome number or by measurement of the size of epidermal cells, particularly the stomata.

While the determination of chromosome number is relatively easy in Papaver from mitotic root tips, the method is time-consuming and can only readily be done by a skilled cytologist with a well equipped cytological laboratory. Epidermal cells can easily be measured with little more equipment than glass slides, cellulose acetate film, and a compound microscope of any kind with no more than a 40X objective lens.

References

Goldblatt, P. (1974) Biosystematic studies in Papaver section Oxytona. Ann. Missouri Bot. Gard. 61, 264-296.

Payne, W.W. (1970) Helicocytic and allelocytic stomata: Unrecognized patterns in the dicotyledonae. Amer. Jour. Bot., 57, 140-147.

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SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM 1/

No. 20

FIELD TEST FOR THEBAINE ANALYSIS 2/

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and

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1/ General note: The views expressed by collaborating scientists in their contribution to the programme are not necessarily shared by the Secretariat.

2/ In the report of the Third Working Group on Papaver bracteatum (document ST/SOA/SER.J/15), it was recommended that a rough screening method should be developed for the determination of thebaine.

3/ This paper was submitted by Professor C.A. Salemink, Head of the Department of Organic Chemistry of Natural Products and Director of the Organic Chemical Laboratory, State University of Utrecht, The Netherlands.

GE. 75-12086

FIELD TEST FOR THEBAINE ANALYSIS 4/

The following method for the detection of thebaine in fresh plant material might be very useful in the research on Papaver bracteatum in order to obtain a rapid and reliable indication of the presence of thebaine.

Minimum amount of fresh plant material

Leaf	:	2 g	(2 leaves of about 15 cm length)
Stem	:	1 g	(1 piece of stem, length 5 cm)
Root	:	1 g	(1 piece of root, average thickness: 0.5 cm, length: 10 cm)
Capsule	:	0.5 g	(1/3 - 1/2 part of one fresh capsule, length: 3-4 cm).

Materials and equipment

1. Merck SiO_2 "Fertigplatten" 15 x 1,5 cm.
2. Glass bottle, containing 250 ml of a saturated solution of concentrated ammonia (specific gravity 0.910) in diethylether.
3. Test tube (15 x 2 cm), containing 1 g of iodine crystals.
4. Test tube (15 x 2 cm), containing a strip of filter paper and 2 ml of "solvent system I" 5/ - toluene:acetone:methanol: ammonia 6 N (40:40:30:2).
5. Test tube (15 x 2 cm), containing a strip of filter paper and 2 ml of "solvent system II" 5/ - ethylacetate:methanol: ammonia 13 N (17:2:1).
6. Pasteur pipettes, watch glasses (diameter 10 cm), pair of scissors, mortar and pestle, test tube (12 x 1,1 cm), containing a solution of 50 mg thebaine in 10 ml diethylether.

4/ Suggestions for modifications which will improve the speed and reliability of the proposed method will be very welcome.

5/ G.C. Jain and M.S. Dahiya, Current Science (1974), 14, 444.

Procedure

1. Cut plant material into small pieces.

2. Macerate with 25 ml of diethylether/ammonia for 3 minutes.

3. Pipette the ether layer into a watch glass and, with gentle swirling, let the solvent evaporate to a small volume 6/.

4. Put two drops of the concentrated extract on a thin layer strip, as shown in Figure 1, in line with two reference spots of thebaine.

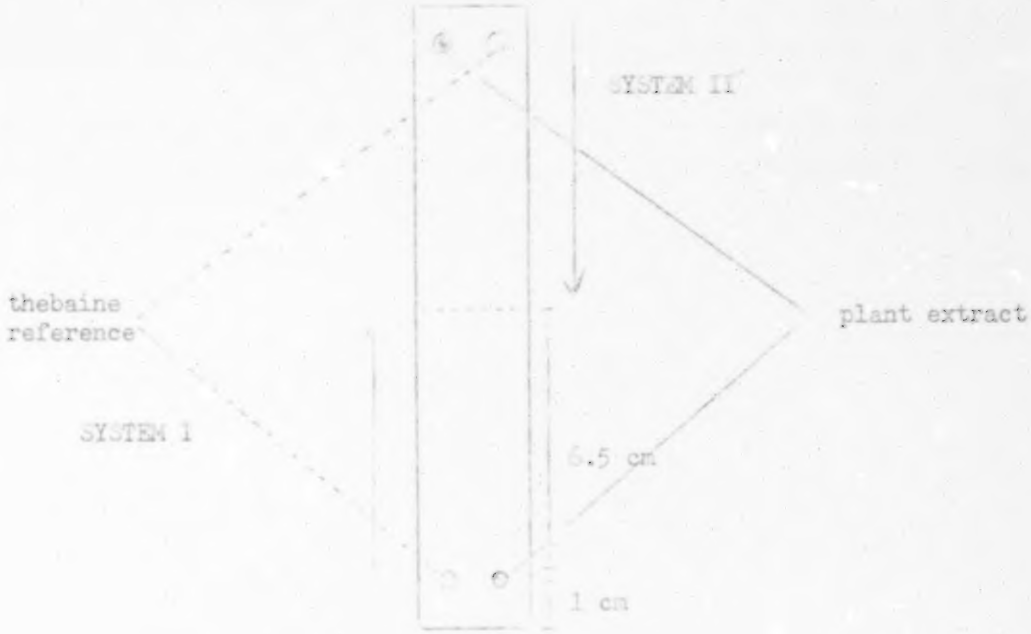

Figure 1

5. Place one of the "spotted sides" of the thin layer strip in solvent system 1. Let the solvent mixture elute until the solvent front reaches the dotted line (Figure 1). Next, remove the strip from the "system I tube" and dry in air for about 5 minutes.

6/ Note: When small droplets of water separate from the ethereal extract, on the watch glass, add some ether, since the application of water to the thin layer plate will severely disturb the smooth elution of the extract spots.

6. Repeat this procedure after turning the strip and use "solvent system II" for the second elution.

7. Visualize the spots in the tube containing the iodine crystals (limit of detection: approximately 1 - 2 micrograms of thebaine; time of visualization at least 3 minutes).

8. When spots from the plant extracts correspond with the thebaine reference spots (Rf 0.15 in system I and Rf 0.50 in system II) the test is considered to be positive.

9. The concentration of the thebaine in the reference spot should be approximately equal to that of the "sample-thebaine". If necessary, repeat the procedure with an "adjusted reference concentration".

The above method was applied to young leaves of P. bracteatum Arya I, grown in The Netherlands and reproducible results were obtained.

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SCIENTIFIC RESEARCH ON PAPAVER BRACTEATUM [1]

No. 21

Rapid semi-quantitative spot test for determination of thebaine and differentiation of Papaver bracteatum from P. orientale and P. pseudo-orientale [2]

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1/ General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.

2/ In the report of the Third Working Group on Papaver bracteatum (document ST/SOA/SER.J/15), it was recommended that a rough screening method should be developed for the determination of thebaine.

GE. 75-12087

The section Oxytona of the genus Papaver consists of three distinct species which occur in Turkey (1), i.e. P. bracteatum (2n=14), P. orientale (2n=28) and P. pseudo-orientale (2n=42). Differentiation between the species has been limited to observing the plant at time of flowering, analyzing plant tissue for alkaloid content, determining the number of chromosomes, and often combinations of these. In each instance problems limit its applicability to species differentiation, e.g. flowering occurs within a limited time frame, alkaloid determination requires somewhat sophisticated equipment, and chromosome determinations require especial expertise and are laborious.

At the Third Working Group on P. bracteatum it was recommended that a rapid field test for thebaine be forthcoming. One such test has already been submitted for publication in the United Nations ST/SOA/SER.J/.. (Küppers, F.E.J.M. and P.G. Vincent. Field test for thebaine analysis, 1975). The test involves the use of thin-layer chromatography using reference thebaine standards and two solvent systems.

The method described herein exploits the fact that the principal alkaloid found in P. bracteatum is thebaine which gives a rather specific colour reaction with molybdate in concentrated sulfuric acid.

Colour was determined using the Inter-Society Colour Council-National Bureau of Standards (ISCC-NBS) Colour-Name Charts Illustrated with Centroid Colours (3, 4). Colours are described using ISCC-NBS designation followed by Munsell renotation (4) in parenthesis.

In 1866, Fröhde introduced his reagent as a test for morphine (5). The reagent is made by dissolving 0.5 g of ammonium or sodium molybdate in 100 ml concentrated sulfuric acid. This reagent should not be confused with "Buckingham's reagent" which contains 10-15 g molybdate per 100 ml. Fröhde's reagent is sensitive to approximately 1 µg of alkaloid. The alkaloids found in the genus Papaver give some specific colour reactions in the presence of a few drops of Fröhde's reagent. For example, morphine plus reagent initially produced a very deep purple red, ISCC-NBS No. 257 (6.8 RP 1.7/8.0) followed by dark olive, ISCC-NBS No. 108 (8.9 Y 2.4/3.1) which faded to medium red brown, ISCC-NBS No. 43 (9. OR 3.4/5.2) and finally to strong green, ISCC-NBS No. 141 (5.8 G 4.4/8.7). Codeine plus reagent gave an initial deep yellow green colour, ISCC-NBS No. 132 (0.9 G 3.5/9.0) followed by a dark olive green, ISCC-NBS No. 126 (8 OGY 2.2/3.6). Papaverine in

the presence of reagent gave a brilliant yellow green, ISCC-NBS No. 116 (4.0 GY 8.2/9.1). Fröhde's reagent plus isothebaine initially produced a dark blue, ISCC-NBS No. 183 (2.2 PB 1.7/5.5) which immediately went to dark olive brown, ISCC-NBS No. 96 (2.6 Y 1.9/2.2). Initial colour of oripavine plus reagent was light olive, ISCC-NBS No. 106 (8.2 Y 5.1/5.6) which turned to medium olive, ISCC-NBS No. 107 (7.6 Y 3.8/5.4). Narcotine plus reagent was grey green at first, ISCC-NBS No. 150 (8.8 G 4.5/1.8) then became dark green, ISCC-NBS No. 146 (6.6 G 2.8/4.6). Orientalidine plus reagent was medium grey ISCC-NBS No. 265 (3.3 GY 5.4/0.1) and alpinigenine was grey violet, ISCC-NBS No. 215 (1.2 P 3.3/3.9). Narceine plus reagent was blue grey, ISCC-NBS No. 191 (8.9 B 5.5/0.9). Thebaine plus reagent gave a deep orange, ISCC-NBS No. 51 (4.1 YR 5.1/11.3). The colour reactions are not specific for alkaloids. Other substances such as aromatic amines, phenols or ethers of phenols yield different colours. However, the colours and sequence of colour change seem to be, in general, specific for each substance.

Method

White enamel spot plates, 9 x 12 cm, having 12 wells were employed throughout. Fröhde's reagent was prepared by dissolving 0.5 g ammonium molybdate in 100 ml concentrated sulfuric acid. Aliquots containing 1 to 120 µg of thebaine were pipetted into the wells. Approximately 10 drops of Fröhde's reagent were added to each well. The colour from the reaction of the known concentration of thebaine was compared to the colour of the unknown and a visual semi-quantitative estimation of thebaine from P. bracteatum could thus be made. Care must be taken to avoid introduction of moisture into the reagent or during the testing itself, particularly under high humidity conditions. Moisture will obviate the colour reactions and, in most cases, is responsible for the sequence of colour changes observed.

This procedure could be used prior to analyses by more sophisticated methods such as gas-liquid chromatography or high performance liquid chromatography when screening extracts for their thebaine content.

Fröhde's reagent can also be added directly to latex from species of Papaver. Latex may be derived from excised leaves, roots or capsules. For qualitative analysis, latex from P. somniferum, P. bracteatum, P. orientale, P. pseudo-orientale and P. alpinium excised leaf tissue were spotted in the wells of the plate and allowed to dry. Ten drops of Fröhde's reagent were added to each well, the plate gently rotated, and the colour development noted. The initial colour reactions were, for P. somniferum latex a deep purple red, ISCC-NBS No. 259 (7.1 RP 2.7/6.0), followed by a medium red brown (described above), and finally a dark olive green (described above). P. orientale latex with reagent gave an initial colour of very dark yellow green, ISCC-NBS No. 138 (0.3 G 1.8/4.3), followed by brown black, ISCC-NBS No. 65 (7.8 YR 0.6/0.9). P. pseudo-orientale with reagent gave an initial colour of very dark red, ISCC-NBS No. 17 (4.2 R 1.2/4.8), followed by a dark red brown, ISCC-NBS No. 44 (9.6 R 1.3/3.6). P. alpinium latex with reagent yielded similar colours as those described for P. pseudo-orientale. P. bracteatum latex with reagent was initially very deep red, ISCC-NBS No. 14 (6.5 R 1.7/8.4), followed by slight red brilliant, ISCC-NBS No. 40 (0.3 YR 3.1/9.9).

Spot plates were prepared by Dr. J. Duke, Plant Taxonomy Laboratory, USDA, ARS, Beltsville, Maryland 20705, as a "blind" test for discriminating between the Papaver species as well as other plant tissue. Plants evaluated were P. bracteatum, P. orientale, P. pseudo-orientale, P. pseudo-orientale c.v. "May Curtis", Eschscholzia californica (California poppy), P. lateritium, Asclepias syriaca (milk weed), Lactuca sativa (lettuce) and Euphorbia sp. (mole plant). Results of the reaction of these plant latex (or sap) materials with Fröhde's reagent are shown in Table 1. In all cases we were able to distinguish the P. bracteatum latex from other expressed fluids with the possible exception of P. fugax. Species differentiation is probably due to the fact that 95-98% of the alkaloids in the P. bracteatum latex consist of thebaine, whereas the other Papaver species contain different alkaloids or thebaine in conjunction with other alkaloids. The effect of the presence of other alkaloids is to quench the slight red brilliant colour typically produced in the bracteatum samples. In addition to the alkaloids, other phenolic or aromatic amine substances are undoubtedly reacting with the reagent and producing specific colours.

A semi-quantitative estimation of thebaine content of the latex may be made in the following manner. A capillary pipette (1-5 µl) is used to collect the latex. The latex may be directly transferred to the spotting well. Some loss will occur due to adhesion of the material to the pipette. During quantitative determinations the pipette plus latex can be dropped into a screw cap test tube containing a given volume of methanol. The test tube containing the methanol together with the pipette and the latex is then agitated for several minutes. An aliquot of the resulting solution, usually 100 µl from a total volume of 1 ml, is placed in the spotting well and allowed to dry. The advantages of the methanol method are rapid dehydration of latex, removal of most substances which might interfere with the colour reaction, and a more quantitative extraction especially if the methanol is made slightly acidic prior to extraction with a volatile organic acid. It should be noted that the volume of latex in the plant will change throughout the day as a function of moisture loss or gain. Therefore, caution must be used when extrapolating data derived from pipetted samples. Leaf tissue subjected to direct application of Fröhde's reagent gave variable colour reaction and is not recommended for thebaine determination.

Thus, a comparatively simple, portable and inexpensive test for thebaine determination is presented. The test also permits the user to distinguish P. bracteatum from P. orientale and P. pseudo-orientale by exploiting the fact that the principal alkaloid in P. bracteatum is thebaine.

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Table 1

Colour reactions of different plant latex or sap with Fröhde's reagent

Plant	Final colour 1/
P. bracteatum latex	slight red brilliant
P. pseudo-orientale latex	dark red brown
P. pseudo-orientale c.v. "May Curtis"	dark red brown
P. fugax latex	dark olive green
P. lateritium latex	dark grey olive green
Eschscholzia californica (California poppy)	dark grey olive green
Asclepias syriaca (milk weed)	grey olive green
Lactuca sativa (lettuce)	brown orange
Euphorbia sp. (mole plant)	brown orange

1/ After exposure to reagent for 5 minutes; refer to ISCC-NBS numbers given in the text for colour designation.